(54) Title: METHOD FOR SEPARATING NUCLEIC ACIDS INTO POPULATIONS

(57) Abstract

A method for at least partially separating nucleic acid molecules in a sample into populations wherein a population is tagged or capable of being tagged with a moiety capable of being immobilised on a matrix, said method comprising contacting the nucleic acid containing sample with a matrix whereby the tagged molecules are captured by the matrix and thereby separated from untagged molecules. The method may be used in a variety of applications, including separating restriction enzyme digestion products, separating linear from circular nucleic acid molecules, in vitro packaging of bacteriophage particles, diagnostic PCR.

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METHOD FOR SEPARATING NUCLEIC ACIDS INTO POPULATIONS

The present invention relates to a method of
separating nucleic acid fragments. In particular, it
relates to a method for separating from a population of
nucleic acid molecules those which are tagged or capable
of being tagged with a moiety which can be immobilised
on a matrix.

Manipulation and handling of DNA is central to most
biotechnology techniques. The manipulation of DNA
typically involves endonuclease digestion using specific
restriction enzymes which cut the DNA into fragments,
followed by purification of the DNA fragments, insertion
of the required fragment into cloning vectors and
transfer of these vectors into non-native hosts for
transcription, and optionally translation, thereby
providing valuable biological information, and/or
expression of the inserted DNA into a product, such as a
therapeutic product. This enables, for example,
eucaryotic proteins to be expressed in bacteria. The
ability to cut and join DNA molecules or fragments is
central to modern biotechnology. Often, these DNA
fragments are generated by the polymerase chain reaction
(PCR), which is now a common tool both in research and
industrial biotechnology. This method enables specific
DNA molecules to be amplified by means of specific short
nucleic acid primers to produce large quantities of DNA
which can then be further manipulated for example using
the aforementioned cloning techniques.

Methods of nucleic acid manipulation which involve
the use of DNA molecules generated for example by means
of the aforementioned restriction enzyme cutting or by
PCR are often inefficient due at least in part to the
presence of unwanted DNA molecules. Such 'unwanted'
molecules include for example vector DNA resulting from
excision, particularly incomplete excision, of an

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inserted DNA fragment from a recombinant molecule,
partially digested restriction fragments or other by-
products of restriction enzyme cutting of DNA molecules,
excess PCR primers, incorrectly ligated nucleic acid
molecules which are the by-products of nucleic acid
manipulation, and PCR products which are the result of
misannealing of a PCR primer with the template nucleic
acid. The quality of primary end product DNA is crucial
for the success of downstream manipulations such as
ligation and transformation of bacterial or eukaryotic
host cells. The ability to separate mixtures of nucleic
acid molecules, such as mixtures of DNA molecules or DNA
fragments into different populations and thereby remove
what is considered to be the 'unwanted' or contaminating
population from the desired or target nucleic acid
molecule would thus enhance the efficiency of further
processing or downstream steps using such generated
nucleic acid molecules.

Methods for purifying nucleic acid molecules as a
class are known in the art. There are however limited
methods available which can separate mixtures of nucleic
acid molecules such as mixtures comprising several
different DNA molecules into different populations.
Generally, these methods rely on separation of nucleic
acid molecules, or fragments, according to size, for
example by means of electrophoresis through agarose or
polyacrylamide gels, followed by purification of the
desired molecule or fragment. These methods have a
number of drawbacks. One limiting factor is the
capacity of the gel itself, which limits the amount of
DNA which can be separated. The DNA needs to be
visualised in the gel, generally by way of staining with
ethidium bromide. Aside from being toxic to the
operator, this can contribute to a reduction in the
quality of the nucleic acid, so that performance in
downstream applications can be poor. Recovery of
nucleic acid molecules fractionated by gel

electrophoresis is also inefficient leading to
significant losses, often of at least 20% of DNA. Gel
electrophoretic methods are also time consuming. DNA is
a fragile molecule and is vulnerable to attack by exo

5   and endonucleases. The comparatively long process of
electrophoretic separation, during which the DNA is
vulnerable to degradation, can thus be detrimental to
the integrity of the DNA, and affect the efficiency of
downstream processes. Such separation methods are thus

10 inefficient and costly. There is thus a need for a new
method of at least partially separating nucleic acid
molecules into different populations. The present
invention provides such a method.

    According to the present invention, there is

15 provided a method for at least partially separating
nucleic acid molecules in a sample into populations
wherein a population is tagged or capable of being
tagged with a moiety capable of being immobilised on a
matrix, said method comprising contacting the nucleic
acid containing sample with a matrix whereby the tagged

20 molecules are captured by the matrix and thereby
separated from untagged molecules.

    This method is much simpler than the aforementioned
electrophoretic separation method and is also quicker.

25 It is thereby more cost effective, has greater all round
efficiency, and does not suffer from the drawbacks of
electrophoretic separation.

    The method relies upon the tagged nucleic acid
molecules being captured, i.e. immobilised or retained

30 on the matrix, thereby effecting a separation from
untagged molecules which remain in solution.

    The method of the invention can be used to separate
untagged nucleic acid molecules of interest (target
molecules) from tagged unwanted nucleic acid molecules

35 or fragments, in which case it is the unwanted nucleic
acid molecules which are captured by the matrix, leaving
the target molecules free in solution. This is

advantageous where the desired target nucleic acid
molecules are intended for downstream processing for
example by further genetic manipulation techniques since
it enables the further steps to be carried out without

5   the need to elute or otherwise detach the desired
nucleic acid molecules from the matrix, and it also
avoids the need, if necessary, to remove the tag from
the nucleic acid molecules. This thus constitutes a
preferred aspect of the invention. The method may

10   however also be used to separate tagged target nucleic
acid molecules of interest from untagged unwanted
nucleic acid fragments, in which case it is the target
molecules of interest which are captured by the matrix
leaving the unwanted nucleic acid molecules in solution.

15   Additionally, the method may be used to collect all
separated nucleic acid fractions for different
downstream purposes.

As used herein, 'nucleic acid molecule' refers to
any nucleic acid molecule, including DNA, RNA, cDNA and

20   hybrid compounds such as compounds comprising nucleic
acids and peptides such as peptide nucleic acids (PNA);
and in the case of DNA, it includes double stranded and
single stranded molecules, and any synthetic DNA or RNA
molecule and hybrid DNA/RNA molecules (ie molecules

25   where one strand is DNA and the other is RNA). 'Target'
or 'desired' DNA refers to the nucleic acid molecule
which is intended to be isolated or separated from other
nucleic acid molecules. In the context of the
invention, 'tag' refers to a moiety which can be

30   attached to, bound to, incorporated in, carried by a
nucleic acid molecule, or be part of the nucleotide
sequence of the nucleic acid molecule, or otherwise
linked to a nucleic acid molecule, and which serves as a
means for capturing the tagged population of nucleic

35   acid molecules from a nucleic acid containing sample in
which one particular population is tagged in this way
and other populations are not tagged. The tag thereby

enables the nucleic acid mixture to be fractionated, with tagged molecules being separated from untagged molecules by means of a retention step using a matrix. The tag may be incorporated into the nucleic acid molecule, i.e. be part of the nucleic acid molecule, for example it may be part of a modified nucleotide and incorporated into the nucleic acid molecule during synthesis, or be a part of the nucleic acid sequence of the molecule, in which case the nucleic acid molecule is itself referred to as tagged, or the tag may be attached or bound to a nucleic acid molecule, for example by post synthetic steps for example by addition of terminal nucleotides, or by binding to a recognition sequence within the nucleic acid sequence, in which case the nucleic acid, without tag attached, is described as being capable of being tagged.

The method may be used to separate or fractionate any of the aforementioned classes of nucleic acid, or mixtures of these. A preferred aspect of the invention comprises the fractionation of DNA molecules or fragments in a sample to at least partially separate the sample into different populations of nucleic acid molecules. Examples of such methods includes the separation of particular restriction fragments from a restriction enzyme digested DNA preparation, for example a PCR generated DNA molecule, or a recombinant DNA molecule, and the "clean-up" of PCR reactions, i.e. the removal of PCR products which are the result of misannealing of primer. The method has other applications also, and may be used, for example, to separate linear and circular nucleic acid, in diagnostic PCR and *in vitro* packaging of bacteriophage.

The moiety used to tag nucleic acid molecules in accordance with the method of the invention may be any moiety which is capable of tagging a nucleic acid molecule and of immobilisation on a matrix. Immobilisation may be either by direct or indirect

interaction with the matrix. The tag may thus alone be
responsible for immobilisation on the matrix, or the tag
may act via an intermediate or linking moiety which is
responsible for interaction with the matrix, such as a
5   binding partner for the tag.

Thus viewed from a further aspect, the present
invention provides a method for at least partially
separating nucleic acid molecules in a sample into
populations wherein a population is tagged or capable of
10   being tagged with a moiety capable of being immobilised
on a matrix either directly or indirectly via a binding
partner for the tag, said method comprising contacting
the nucleic acid containing sample with a matrix, or,
where the tag interacts indirectly with the matrix by
15   means of a binding partner, with a binding partner for
the tag and with a matrix, whereby tagged molecules are
captured by the matrix and thereby separated from
untagged molecules.

The nature of the tag will depend at least in part
20   upon the molecules which are to be separated and on the
matrix used. Examples of suitable tags include moieties
which can be incorporated into a nucleic acid molecule,
for example ligands for example biotin, fluorescein or
steroids or steroid like molecules such as digoxygenin,
25   or which can be used to modify individual nucleotides
within a nucleic acid molecule, and moieties such as
proteins, for example proteins which have an affinity
for a particular binding site within a nucleic acid
molecule. The tag may thus be introduced into the
30   nucleic acid molecule during its synthesis, for example
by means of a tagged nucleotide, or after synthesis for
example by addition at one end of tagged nucleotides
e.g. by means of an enzymic reaction. Depending on the
matrix used, the tag may interact directly with the
35   matrix, or the tag may indirectly interact by way of a
binding partner for the tag which serves to immobilise
the tag on the matrix and thus acts as a linking agent.

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The binding partner may itself interact directly with the matrix, or it may interact by way of a further linking agent, in which case the tagged molecules may go through sequential or concurrent binding steps to enable the tagged molecule to be captured by the matrix.

In one embodiment, the tag may be a small molecule ligand. In this case, the tagged nucleic acid molecule may be immobilised on the matrix in the method of the invention by means of a binding partner to the ligand, which may be immobilised on the matrix in the form of a binding partner derivatized matrix, or which may serve as a separate linking group to immobilise the tag on the matrix.

In one embodiment of such a method, the nucleic acid sample is first contacted in solution with binding partner for the ligand tag, which binds only to the tagged nucleic acid molecules, and the binding partner bound tagged nucleic acid molecules are then extracted by means of a matrix with affinity for the binding partner. In another embodiment, the binding partner is first immobilised on the matrix, and is then contacted with the nucleic acid sample, retaining on the membrane only tagged nucleic acid molecules. In this embodiment, the binding partner may be immobilised onto the matrix using conventional methods appropriate for the type of matrix and the binding partner, including direct chemical bonding such as covalent bonding, adsorption, or by affinity binding.

One example of a ligand tag which may be used in the invention is biotin. Others are known in the art. Where biotin is used as the ligand, the binding partner is avidin or streptavidin, and the matrix may be one which has an affinity for proteins and is thereby able to capture streptavidin or avidin and any molecules to which (strept)avidin is bound. Avidin and streptavidin may each be used as the binding partner for biotin, and where in the following reference is made to

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streptavidin, the bacterial protein, it will be
understood that avidin could also be used. Biotin can
be readily incorporated into nucleotides, and indeed
biotinylated nucleotides are available commercially. We
5   have also found that the use of a biotin tag is very
efficient in the method of the invention. Biotin
accordingly represents a preferred tag for the method of
the invention.

In one embodiment where biotin is used as the
10   ligand, biotinylated nucleic acid molecules may first be
incubated in solution with streptavidin, whereby
streptavidin as binding partner will bind to any biotin
containing nucleic acid molecules and form a binding
complex. These tagged molecules with streptavidin
15   attached are then subsequently immobilised by means of a
matrix capable of selectively immobilising proteins,
whilst not being capable of immobilising nucleic acids
at least under the conditions used, thereby separating
out from the sample the biotin containing nucleic acid
20   molecules.

In another related embodiment where biotin is used
as the tag, the matrix itself will have streptavidin
bound or attached to it. In this case, when the nucleic
acid sample in solution is contacted with the matrix,
25   biotin-tagged molecules will be retained, leaving
untagged molecules free in solution.

In another embodiment, the tag may be a ligand such
as fluorescein or digoxigenin or an antigen. These tags
may be captured on the matrix by means of binding
30   partners to these tags, for example an antibody to the
tag, either polyclonal or monoclonal, or a fragment of
such an antibody. Where the ligand is a steroid, the
capturing means may be either an antibody, or a fragment
thereof, or a receptor for the steroid, or a fragment
35   thereof with steroid binding properties. These
capturing means may be utilised in a similar fashion to
the aforementioned use of streptavidin, with a matrix

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which has an affinity for proteins.

In a further embodiment of the invention, the tag
may be a protein, preferably a nucleic acid binding
protein, which has a specific recognition sequence
5   within the nucleic acid molecule to be tagged. Examples
of such nucleic acid binding proteins include the
transcription factor AP-1 which binds to the AP-1
recognition sequence, the myb protein which binds to a
specific short recognition sequence, and the lacI
10   repressor protein, which binds to a lac operator
sequence.

In a related embodiment, the tag may be viewed as a
nucleic acid sequence or sequences within the nucleic
acid molecule such as a specific recognition sequence.
15   Such sequence tags may have affinity for a protein which
can be bound to a matrix. Examples include the
aforementioned AP-1 recognition sequence, to which AP-1
as binding partner may bind and thereby effect binding
to a protein-binding matrix. Similarly, the myb protein
20   as binding partner may bind to a specific short
recognition sequence as tag, and the lacI protein as
binding partner may bind to the lac operator sequence as
tag.

In such embodiments of the invention, a sample
25   containing nucleic acid molecules which include the
protein recognition sequence may be first further tagged
in solution by contact with the protein recognised by
the specific sequence, and then the sample is contacted
with the matrix whereupon tagged molecules are retained
30   on the matrix leaving untagged molecules i.e. molecules
without bound protein in solution. Alternatively, the
DNA binding protein may be immobilised on the matrix,
and then used to capture the nucleic acid from solution,
in a similar way to the above described methods which
35   use streptavidin.

In each of these embodiments, a protein is involved
in capturing a population of nucleic acid molecules,

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either as the tag itself, or as the binding partner for
the tag (such as antibody, or streptavidin). This is
advantageous because it enables protein receptive
materials to be used as the matrix, preferably materials
5   which have selective binding for proteins and thus which
do not bind nucleic acids, at least under the conditions
used, thereby ensuring that untagged molecules are not
captured by the membrane and sequestered from the
sample. The protein may be captured by the matrix and
10   bound to it by a variety of interactions, including
ionic interaction, hydrophobic interaction and affinity
binding.

The matrix may take any convenient physical form,
and many are known in the art, for example sheets, gels,
15   filters, membranes, fibres, tubes, microtitre plates,
columns, particles, and may be particulate or porous.
Porous materials such as filters and membranes are
convenient for separation methods according to the
invention, either for filtering away unwanted tagged DNA
20   or for collection of wanted tagged DNA. Examples
include samples where an untagged population is intended
for further downstream processing, and where the tagged
nucleic acid population, constituting the 'undesired'
population, may be captured from solution by the
25   membrane, since it is straightforward to process the
sample by filtration through the membrane offering an
effective and rapid capture method and simultaneously
fractionating into the filtrate the untagged nucleic
acid population, thus offering a straightforward route
30   into the next manipulation stage. Porous materials such
as membranes thus constitute a preferred matrix for use
in the invention.

Porous matrices may thus be conveniently used for
filtering away unwanted tagged nucleic acid molecules or
35   for collection of wanted tagged nucleic acid molecules.
Such matrices may be used as part of a device for a
single or multistep separation, or as part of other

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steps, such as for detecting, assessing or quantitating
DNA or any other product in a downstream reaction
stream. These porous matrices may be incorporated into
separation devices such as centrifuge vials, microtitre
plates, cartridges or syringes, and, depending on the
sample and the downstream processes to be operated, one
or more of such devices may be provided in a serial
manner. Such devices may be handled manually,
semiautomatically or in fully automated fashion.

In one embodiment, a NycoCard™ may be used. Where
the tagged nucleic acid fragment is the sequence or
molecule to be detected, it may, after binding for
example to a protein with affinity for the tag be
entrapped directly in a protein binding membrane
retained in a NycoCard device. Such a device would
include an appropriate membrane with an absorbent pad
such as cellulose paper placed on one side to enhance
passage of the liquid sample through the membrane. In
one embodiment, an impermeable sheet may be placed over
the other side of the membrane and holes may be provided
to permit application of samples to the membrane in the
case where multiple samples are to be analysed. Where
the tagged sequences are to be eliminated, and untagged
nucleic acids collected, a protein binding filter may be
used as a prefilter, placed over a nucleic acid binding
filter mounted in the NycoCard device so that unwanted
tagged molecules are retained on the prefilter, and
desired untagged molecules retained on the nucleic acid
binding filter.

The matrix may be composed of a variety of
materials known in the art for the purpose, including
polymeric materials for example cellulose, polystyrene,
agarose, latex, which may be derivatized or modified to
provide means for capturing the tag itself, or for
capturing the binding partner for the tag which acts as
a linking agent between the tag and the matrix. Thus
for example, the material may be treated eg by coating

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with a substance having an affinity for the tag, or the
binding partner or linking agent used to mediate capture
of the tag. Preferably, the matrix will have
specificity for the tag or the binding partner for the

5   tag, as compared to nucleic acid, so that untagged
molecules are not retained by the matrix. In a
preferred aspect of the invention, the capturing process
involves a protein, either where the tag is a protein,
or is a substance which has a proteinaceous binding

10   partner which serves to link the tag to the matrix, and
the matrix is thus of a protein receptive nature.
Examples are known in the art and include known protein
binding matrices, coated for example with polymers
having a specific affinity for proteins, at least under

15   the conditions used. The matrix may be substituted with
or carry the binding partner for the tag according to
methods known in the art, such as described in
WO90/04786 including direct chemical bonding such as
covalent bonding, adsorption, or by affinity binding.

20   Particularly preferred are membranes comprised of
protein binding polymers, such as those described in
WO98/23630,EP-0524800 and EP-0580305, for example
Centriflex (TM) marketed by Edge Biosystems, USA.
       The separation method of the invention is

25   particularly convenient where a sample is to be
fractionated, and untagged nucleic acid molecules in
solution are to be collected for further downstream
processing. The method may however also be used where
it is the tagged nucleic acid molecules which are to be

30   collected for further processing. When tagged molecules
are those to be collected for downstream work, the
molecules will need to be released from the matrix, and,
depending on the capture method used, may also need to
be released from the binding partner for the tag. The

35   release method used may depend upon the nature of the
tag, and its binding partner, and the type and strength
of their mutual binding forces.

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The reaction conditions chosen for the release step may also be selected so as to prevent the released tagged nucleic acid from rebinding to the matrix or to its binding partner. Examples of such methods are known
5   in the art. Thus for example, chemical or physical conditions may be changed so as to aid release of tagged nucleic acid molecules from the matrix -bound complex. Examples of chemical methods include altering ionic strength or pH, addition of chelating agents, and the
10   use of competing free tag molecules, or molecules chemically related thereto, molecules comprising tags or tag like moieties, molecules or ions which change the conformation of the binding partner and thereby reduce, eliminate or modify the tag-binding partner interaction,
15   addition of detergents or dissociating agents, or by enzymatic treatment. Examples of physical methods include changes in temperature, sonication, vibration. Any combination of these physical and chemical methods may be used.
20   Depending on the strength of the interaction between the tag and its binding partner, it may be possible to release the tag or the tagged molecule from the binding partner or matrix by means of adding excess tag which competes with the tagged DNA for attachment
25   sites to the binding partner for the tag or to the matrix, and the released tagged DNA may then simply be washed off. Thus where the tag is fluorescein, and the binding partner is an antibody to fluorescein, fluorescein-DNA may be released from the matrix by
30   addition of free fluorescein in solution. Where however the tag-binding partner interaction, binding partner-matrix interaction or tag-matrix interaction is strong, addition of free tag is not always effective at disrupting the interaction. In this case, other methods
35   such as by degrading the binding partner for example by means of pH or enzymes in such a way that it is released from the matrix and/or the tag may be used. Release may

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also be effected by disruption of the matrix into a form
unable to bind proteins, such as by means of chemical
agents, so that the protein-DNA or protein-tag-DNA
complex is released, or by chemical means which disrupt
the interaction or otherwise effect the affinity between
protein and matrix.

The biotin-streptavidin binding pair is one where
there is a particularly strong interaction and thus is
not susceptible to disruption by means of addition of
free biotin. Thus where the tag is biotin, and the
tagged DNA is captured on the matrix by means of avidin
or streptavidin, biotinylated DNA cannot be released
from the membrane by addition of free biotin. This
method can however be used where the tag is a biotin
derivative with lower binding affinity than biotin to
streptavidin, in which case the tagged DNA may be
released from the matrix by addition of free biotin.
This may compete with the biotinylated DNA for
attachment sites to streptavidin and the released
biotinylated DNA may simply be washed off the matrix.

Depending on the tag used, it may be necessary to
incorporate a step whereby the tagged DNA which is
intended for downstream processing is released from the
binding partner for the tag which serves to immobilise
the tagged DNA on the matrix. Examples include addition
of chemical substances or ions or by applying physical
conditions capable of reducing the binding force between
the tag and its binding partner and then collecting the
released DNA.

The method of the invention can be used in a number
of different applications, including analytical,
preparatory and diagnostic uses, examples of which are
presented below. Other applications will be apparent to
those skilled in the art.

An important application of the invention is in the
cutting and ligating of DNA molecules, such as in the
separation of particular restriction enzyme digestion

products, and the separation of ligated, circular DNA
molecules from other products of ligation reactions.

    Thus one application of the invention is in the
manipulation of restriction enzyme digested fragments of
5   DNA particularly where a specific fragment is required
for further manipulation which thus needs to be
separated from other products of the enzyme reaction.
The separation method enables a population of linear DNA
molecules which are tagged at one or both ends to be
10   separated from untagged molecules. The tagged molecules
may be tagged during synthesis, for example using tagged
nucleotides such as biotinylated nucleotides, e.g. in
the form of biotinylated primers, or the molecules may
be tagged enzymically by end-labelling methods known in
15   the art.

    Thus viewed from a further aspect, the present
invention provides a method for at least partially
separating a mixture of restriction enzyme digested
fragments of DNA wherein the starting material is a
20   linear DNA molecule which is tagged or capable of being
tagged at or near one or both ends with a moiety capable
of being immobilised on a matrix, said method comprising
subjecting the DNA molecule to restriction enzyme
digestion followed by contacting the sample with a
25   matrix whereby the tagged molecules which originate from
an end of the starting material are captured by the
matrix and are thereby separated from untagged
molecules.

    This method is particularly well suited to
30   separating digestion products of PCR produced DNA
because of the way the synthesis works. In the PCR
method of DNA amplification, two specific
oligonucleotide primers are used, one of which is
complementary to and therefore hybridises to the 5' end
35   of the coding strand and one of which is complementary
to and therefore hybridises to the 5' end of the
noncoding strand so that in the presence of appropriate

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DNA polymerase enzymes, a full length copy of the target
sequence can be synthesised. This copy will have the
primer oligonucleotide incorporated into each of the two
strands of the synthesised DNA, at the 5' end of each
5   strand. Such PCR synthetic methods are often used to
prepare specific DNA molecules or fragments for
subsequent genetic manipulation, where the specific
fragment of interest can be obtained by restriction
enzyme cutting of a longer PCR product. As previously
10   mentioned, these subsequent manipulations are often
inefficient because of the presence in the subsequent
steps such as ligation mixtures of other products from
the restriction enzyme digest such as partially digested
products and undigested DNA molecules. In circumstances
15   where it is an internal fragment of the full length PCR
product which is the product of interest for downstream
processing, the 'unwanted' by-products of the
restriction enzyme digest will include at least one
terminus of the full length PCR product, the method of
20   the invention can be used to effect an at least partial
separation of the product of interest from end-
containing fragments, and undigested molecules, by
utilising tagged primers for example biotinylated
primers. In this way, tagged nucleic acid molecules can
25   be removed from a nucleic acid sample, leaving in
solution a sample containing the desired internal
restriction fragment which, since it does not include a
terminus for example incorporating a PCR primer, will
not be tagged.
30   A further aspect of the invention pertinent to PCR
methodology is in the so-called "clean up" of the
products of the PCR reaction, i.e. the removal of
unwanted products which include products which are the
result of misannealing of the primers. The larger the
35   nucleic acid template is, the more this can be a
problem. This method has utility where there are unique
restriction enzyme sites or other cleavable sequences,

at or close to the ends of the nucleic acid sample to be
amplified in the PCR reaction.

The method involves the use of PCR primers which
are tagged, or capable of being tagged, and which are
5   complementary to the ends of the sequence to be
amplified and thus are capable of hybridising to the
template nucleic acid and which also overlap only
partially with each of the unique restriction sites.
With the use of primers which are complementary to and
10   hybridise to the full restriction enzyme recognition
sequence within the template nucleic acid, every nucleic
acid molecule produced by the PCR reaction will carry
the restriction enzyme recognition site irrespective of
whether or not the primer has annealed to the desired
15   sequence or whether the PCR product is the result of
misannealing of the primer. However, using primers
which hybridise to only part of a restriction enzyme
recognition site in the template, it is only in the PCR
products which derive from the intended annealing where
20   the restriction enzyme recognition site is reconstituted
in the PCR product. Thus by using tagged primers, or
primers which are capable of being tagged, it is
possible to clean up PCR reaction products by performing
the PCR reaction, and subsequently cleaving or digesting
25   the PCR reaction products with the particular
restriction enzymes specific for the said unique
restriction recognition sites. Upon such restriction
enzyme digestion, it is only the correct PCR products
which are cleaved and which, because cleavage resuts in
30   removal of the primer and tag, can be separated from the
unwanted PCR extension products which, as they are not
cut by the restriction enzymes, still retain the tag.

Thus viewed from one aspect, the present invention
provides a method for at least partially separating the
35   correct and desired products of PCR amplification from
PCR products which result from incorrect annealing of a
PCR primer to template, wherein the template nucleic

acid molecule comprises a unique restriction enzyme
recognition site at or towards an end of the template,
and a PCR primer which is tagged or capable of being
tagged is complementary to a sequence on the template
5   which extends partially into the unique restriction
site, the method comprising amplifying the template by
means of PCR, digesting the PCR products with the
restriction enzyme specific for the said unique
restriction enzyme recognition site, and contacting the
10   resulting product with a matrix capable of sequestering
the tag whereby tagged nucleic acid molecules are
captured by the matrix and thereby separated from
untagged molecules.

        In this context, unique restriction enzyme site
15   refers to restriction enzymes which have only a single
recognition site within the template nucleic acid
molecule, but also includes the special case where the
same restriction site may be present at or towards each
end of the template.

20   In one embodiment, the template nucleic acid
molecule to be amplified by PCR incorporates only a
single unique restriction site at or near only one of
the ends. When the reaction products are treated with
this enzyme this results in a partial "clean-up" of the
25   PCR products the degree of which is dependent at least
in part upon the extent to which the primer which
anneals to the region of the template which includes
this unique site is responsible for incorrect annealing.
In this embodiment, it is the primer which extends into
30   the unique restriction enzyme site which will be tagged
or capable of being tagged. In a preferred embodiment,
the template to be amplified by PCR incorporates a
unique restriction enzyme site at or near to each end.
These sites may be sites for the same restriction
35   enzyme, i.e. a restriction enzyme which cuts the
template and the PCR copies twice, once at each end, or
they may be sites for different restriction enzymes,

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provided that each of said enzymes has only a single
recognition site within the template, and that the two
sites are one at each end of the template.

In this method, the location of the or each unique
restriction site in relation to the terminus of the
nucleic acid template depends, at least in part, upon
the particular restriction enzyme, since different
enzymes have different minimum distances from the
terminus for efficient restriction cutting. These
preferences are known in the art, and are described for
example in the catalogues of manufacturers of
restriction enzymes. In general, the restriction site
will be at least one base from the terminus. The choice
of enzyme and its location may be readily determined by
the skilled person based on knowledge in the art and
according to manufacturer's information. In general,
the restriction site will be located at least the
minimum distance from the terminus for efficient cutting
by the corresponding enzyme, although the site can be
further away from the terminus. Examples of this
minimum distances are at least one base from the
terminus for BamHI and at least six bases from the
terminus for NdeI. Minimum distances may be ascertained
from the published catalogues of suppliers of
restriction enzymes, for example the New England Biolabs
Catalogue 1999, and from Moreira and Noren, Biotechiques
19, 56-59 (1999).

Thus by using or constructing by means of
recombinant DNA techniques known in the art a template
comprising a unique restriction enzyme site at or near
each end, it is possible to remove the unwanted PCR by
products which are the result of mismatched annealing of
PCR primers at locations on the template other than
those intended. Where both primers are tagged or
capable of being tagged, all PCR products will initially
be tagged at both ends. By subjecting the PCR products
to restriction enzyme digestion with the aforementioned

restriction enzymes which have unique recognition sites
towards each end of the template, either simultaneously
or sequentially, molecules which are uncut, or cut at
only one end will retain the tag, as will the termini
5   from the correct PCR product which have been created by
the restriction enzyme cutting; these may be separated
from the desired product which will have had both
primers removed and thus no longer contain a tag or a
moiety capable of being tagged. This has the advantage
10   that expenditure of large amounts of time and materials
to fine-tune the PCR reaction is avoided, as is the need
for gel electrophoresis or other methods of separating
PCR products by size. An at least partial "clean up"
can however be achieved if only one of the two PCR
15   primers is tagged, or capable of being tagged.
        Conveniently, the tag may be biotin, in which case
the PCR products, before or after restriction enzyme
digestion are contacted with streptavidin, and then the
tagged molecules with streptavidin bound are separated
20   by protein-binding matrices from untagged molecules.
Alternatively, streptavidin may be carried on the matrix
itself as previously described.
        In this particular embodiment of the invention, the
tag may be attached at any position on the primer, so
25   long as the 3' end of the primer is available for
elongation by the PCR polymerase. Conveniently, the tag
may be attached at the 5' end of the primer, since
adding a tag at this position does not pose synthetic
difficulties. In addition, without wishing to be bound
30   by theory it is believed that a tag placed at the 5' end
of the primer will be less likely to interfere with the
activity of the PCR polymerase, and also, that it may be
better situated for capture in any of the capture
methods described herein.
35   An example is shown below:
5'-tttactggatcctag------ttacgtacattaatcgg-3'
3'-aaatgacctaggatc------aatgcatgtaattagcc-5'

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This fragment has two unique restriction sites, one for BamHI at the "left" (ggatcc) and one for AsnI at the "right" (attaat). To amplify this fragment in the usual manner, two primers are usually made, optionally labelled with biotins at their 5'-ends, as illustrated below:

BamHI primer: 5'-biotin-tttact**ggatcc**tag-3'
AsnI primer: 5'-biotin-cc**gattaat**gtacgtaa-3'

Using these primers in a PCR reaction will typically produce a number of products, mainly due to misannealing of the primers during PCR.

The BamHI and AsnI primers fully implement the nucleic recognition sequences in their sequences. That has the consequence that every DNA fragment produced in the PCR reaction by these primers has the BamHI and the AsnI restriction enzyme recognition site. All fragments produced in the PCR reaction will be able to be cut by the BamHI and the AsnI enzyme, both the correct (ie desired) ones and by-products.

However, by the use of slightly shorter primers in this embodiment of our invention, we can very efficiently remove all unwanted by-products.

Example of primers which can be used are:

BamHI primer short: 5'-biotin-tttact**gga**-3'
AsnI primer short: 5'-biotin-cc**gatt**-3'

These primers only partly overlap the recognition sites for the restriction enzymes. Therefore, only by annealing to their intended nucleic sites, they will form complete enzyme recognition sites. As a consequence, only correct (or desired) fragments produced from the PCR reaction are able to be cut by both restriction enzymes.

The four possible groups of PCR products which will

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result by the use of the primers (B=biotin label) are as
follows:

```
5'-Btttactggatcctag------ttacgtacattaatcgg-3'
```
5 `3'- aaatgacctaggatc------aatgcatgtaattagccB-5'`
(Correct fragment - two restriction sites)

```
5'-Btttactggtag------ttacgtacattaatcgg-3'
3'- aaatgaccatc------aatgcatgtaattagccB-5'
```
10 *(Incorrect fragment - only one restriction site (AsnI))*

```
5'-Btttactggatcctag------ttacgtacaatcgg-3'
3'- aaatgacctaggatc------aatgcatgttagccB-5'
```
(Incorrect fragment - only one restriction site (BamHI))

15
```
5'-Btttactggatag------ttacgtacaatcgg-3'
3'- aaatgacctatc------aatgcatgttagccB-5'
```
(Incorrect fragment - No restriction sites)

20 Cutting by AsnI and BamHI gives the following fragments:

```
5'-Btttactg     gatcctag------ttacgtaca     ttaatcgg-3'
3'- aaatgacctag     gatc------aatgcatgtaatt     agccB-5'
```
(Correct fragment - two restriction sites- both biotins
25 *removed)*

```
5'-Btttactggtag------ttacgtaca     ttaatcgg-3'
3'- aaatgaccatc------aatgcatgtaatt     agccB-5'
```
(Incorrect fragment - only one restriction site (AsnI)-
30 *only one biotin removed)*

```
5'-Btttactg     gatcctag------ttacgtacaatcgg-3'
3'- aaatgacctag     gatc------aatgcatgttagccB-5'
```
(Incorrect fragment - only one restriction site (BamHI)-
35 *only one biotin removed)*

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```
5'-Btttactggatag------ttacgtacaatcgg-3'
3'- aaatgacctatc------aatgcatgttagccB-5'
```
(Incorrect fragment – No restriction sites – no biotins removed due to no restriction)

In a mixture of all the fragments mentioned above, only one fragment, the correct one, will be without biotin attached. By adding streptavidin to the fragments, and contacting the reaction products with a protein binding matrix, for example by passage through a centriflex membrane, only the correct fragment will not be sequestered, and , in the case of a centriflex membrane, will pass the membrane unhindered.

The difference between using the short primers (ie primers which do not completely extend into the restriction enzyme site on the template) and the long primers (which incorporate the complete restriction enzyme site) is evident, since, in the case of long primers, all fragments, even the erroneous ones, would have the restriction-sites for AsnI and BamHI introduced. In that case, it would not be possible to distinguish between correct and erroneous products of the PCR reaction.

The method may also be used to separate restriction enzyme digestion products of DNA molecules which are either linear or have been linearised and which are tagged at one or both ends by means of end-labelling methods such as those which are known in the art, for example by way of enzymes. One example is the enzyme terminal deoxynucleotidyltransferase (TdT)which can be used alone or together with DNA polymerase I (Klenow fragment) to add tagged nucleotides, for example biotinylated or fluorescein or digoxygenin labelled nucleotides to the free hydroxyl groups at the 3' ends of a linear DNA molecule, thereby adding a tag to the 3' ends. In the case where the linear DNA to be subjected to restriction enzyme digestion is blunt ended or has

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protruding 3' ends, then only TdT is needed to tag the
3' ends. Where however the 5' end is protruding, and
the 3' end is recessed, an enzyme such as Klenow
fragment is additionally needed to fill in the recessed
5   end to enhance efficiency.

Where the tag is biotin, the sample may first be
contacted with streptavidin in solution, and then the
sample is passed through a protein binding membrane
which binds to streptavidin and thereby retains the
10 unwanted, tagged, nucleic acid molecules, leaving the
desired fragment in solution and in a form suitable for
further manipulation.

This represents a significant advance over current
procedures which are labourious and involve the
15 separation of the restriction enzyme digestion mixture
on an agarose gel, visualising the fragments by ethidium
bromide staining, excising the DNA fragment of interest
and purifying it from the gel which is a much more
lengthy procedure and because of this involves a greater
20 risk of exposure of the DNA to nuclease activity.

A further application of this aspect of the
invention is in the field of restriction enzyme
digestion of PCR amplified products. Thus there are
circumstances where it is desirable to carry out partial
25 restriction enzyme digestion, ie with limited amounts of
enzyme. Restriction enzyme preparations often contain
small amounts of endonuclease which can degrade the ends
of newly cut DNA, which can reduce the quality of the
fragment of interest, and cause difficulties in further
30 manipulation steps such as ligating the DNA, for example
into an expression vector. Such problems caused by
contaminating nucleases can however be minimised by
using limited amounts of restriction enzyme and reducing
the incubation periods. A drawback however is that the
35 resulting digest will include unwanted byproducts of the
restriction enzyme digestion, such as only partially cut
molecules or uncut molecules. The method of the

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invention however enables partial digestion to be
exploited, by synthesising the substrate for restriction
enzyme digestion by PCR using tagged eg biotinylated
primer DNA so that any DNA molecule or fragment which
5   includes at least one terminus (such as undigested
molecules, or partially digested molecules) can be
removed or sequestered by means of an appropriate
matrix, leaving a solution enriched in the internal
restriction fragment of interest.

10   A further application of the tag-based separation
method of the invention is in diagnostic PCR. The use
of PCR for detecting mutations including mutations which
differ by as little as only a single nucleotide such as
occurs in sickle cell disease is known. In some cases,
15   the size of a PCR fragment will be indicative of the
presence or absence of a particular mutation. Usually,
however, diagnostic PCR techniques generally require
further techniques following the initial PCR on the
patient sample in order to diagnose whether or not the
20   mutation is present in the sample, for example
restriction analysis and/or sequencing. These can be
costly since they often require expensive chemicals such
as peptide nucleic acids and also time consuming,
contributing to a delay in ascertaining the medical
25   status of the patient. The existing methods are
considerably simplified by using the tag-capture method
of the invention by means of PCR reactions using at
least one PCR primer which is tagged, or capable of
being tagged. By using tagged primers no such further
30   steps are needed, and the presence of a mutation can be
detected using the PCR amplification step alone.

            Thus the method of the invention when applied to
diagnostic PCR is able to replace the need to sequence
PCR products in accordance with current diagnostic
35   methods. In this embodiment, the primer for annealing
to and extending the 3' end of the sample is tagged, or
capable of being tagged in such a way that it retains a

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3' OH group and can thus be elongated in a PCR reaction, and is specific for the mutant nucleic acid; in other words, it hybridises to the sequence in the nucleic acid which is altered in the disease state. Alternatively,

5   the 3' primer may hybridise to the normal nucleic acid sequence.

In one embodiment, the method may be used to detect single base mutations in which case the 3' end of the primer corresponds to the mutated base. In the case of

10   multibase mutations, the 3' end of the primer may correspond to any one of the mutated bases.

Thus, where the mutation is known, in one version of this embodiment, two PCR reactions are carried out on the sample DNA, each uses the same primer for the 5' end

15   of the sample, but with a different 3' primer, one hybridising to and capable of producing an extension product corresponding to the 'normal' DNA, and one complementary to and specific for the mutant nucleic acid sequence and thus capable of producing an extension

20   product corresponding to the mutant sequence. In the case of PCR of a mutated target using the tagged primer appropriate for the mutated DNA, PCR amplification products will incorporate the tag whilst in the PCR reaction using the 'normal' primer there will be a

25   mismatched base at the 3' end of the primer which will be removed by the PCR polymerase, such as Taq polymerase, and, if the 3' base is tagged, its removal will remote the tag or the moiety capable of being tagged which it bears. The PCR product will thus not

30   incorporate the tag. Thus it is only the PCR reaction products of the mutant nucleic acid which are formed by extending the mutant primer which are tagged, or capable of being tagged, and which can be detected using any of the aforementioned methods.

35   The converse situation arises in the case of PCR extension of 'normal' DNA which does not contain the specific mutation to be detected. In this case, it is

the PCR products obtained using the mutant primer where
the 3' nucleotide of the primer is mismatched and
accordingly removed together with the tag which cannot
thus be detected in the full length reaction product
whilst the product using the 'normal' primer will
include the tag or the moiety capable of being tagged
which can then be detected.

In this embodiment, the tag or moiety capable of
being tagged is on or is attached on or to the 3' base,
in such a way that the 3' OH is still extendable by PCR
polymerase.

Thus viewed from a further aspect, the present
invention provides a method of diagnostic PCR in which a
test sample is separately subjected to PCR reactions, in
which the mutation, if present, is in the sequence to
which the 3' primer is complementary, a first PCR
reaction using a 3' primer complementary to the normal
target nucleic acid and a second PCR reaction using a 3'
primer complementary to the mutant target, wherein the
3' nucleotide of the mutant primer corresponds to one of
the nucleotides which is mutated in the mutant nucleic
acid, each of said 3' primers bearing a tag or being
capable of being tagged on the 3' nucleotide of the
primer, wherein the presence or absence of the tag in
the PCR reaction products is detected.

In its simplest embodiment, the 3' primers are
tagged, for example with biotin, which may be detected
using any of the methods described herein. Examples of
methods of detecting biotinylated PCR products include
the addition of streptavidin and filtration through a
protein binding membrane where retained DNA is detected,
or addition of streptavidin-coated gold particles, such
as described in EP-0564494, and passage through a
membrane such as a nitrocellulose membrane, for example
as in the aforementioned NycoCard™ system. Optionally,
but preferably for smaller PCR fragments, the membrane
may be coated with polylysine to improve the DNA binding

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capacity. In such detection methods, the PCR reaction
products will all bind to the membrane but it is only
the biotinylated reaction products that have gold
particles attached and which accordingly give rise to a
5   detectable signal. In an alternative method,
streptavidin may be added to the PCR reaction products
and the mixture passed through a Centriflex membrane.
If no DNA is detectable in the material passing through
the membrane, the DNA will have been retained on the
10   membrane, and thus will have been biotinylated.

    In circumstances where the primer is not itself
tagged, but is capable of being tagged, the tag will
need to be added prior to the detection step.

    In an alternative embodiment, a single PCR reaction
15   may be carried out using a tagged primer specific for
either of the normal or mutated sequences, with the
presence of the tag in the PCR products being indicative
of the presence or absence of the mutation, depending
upon whether the primer is specific for the normal or
20   the mutant DNA. Thus if the tagged primer is specific
for the normal DNA, the PCR product with normal DNA will
be tagged, whilst that with the mutant DNA will not, and
if the tagged primer is specific for the mutant DNA, the
PCR product on normal DNA will be untagged whilst the
25   products of PCR with a mutant DNA will be tagged and
thus detectable. Thus whilst carrying out duplicate PCR
reactions gives more certainty, a single PCR at least
gives an indication of the presence or absence of the
mutation.

30   Thus viewed from a further aspect, the present
invention provides a method of diagnostic PCR of a
mutation in a nucleic acid molecule in which the
presence or absence of a mutation in a nucleic acid
sample is detected, wherein a 3' primer specific for
35   either the normal or mutant nucleic acid is used,
wherein said primer is complementary to a region of the
nucleic acid where there is a base difference between

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the normal and mutated DNA with the 3' terminus of the
primer corresponding to the position in the sample where
there is a difference between the normal and mutant, the
primer being tagged or capable of being tagged, whereby

5   the presence or absence of the tag in the PCR product is
detected.

It is also possible to carry out at least a
preliminary diagnosis using a 3' primer which is
specific for the normal DNA.

10   The present invention accordingly provides from a
further viewpoint a method of diagnostic PCR in which a
test sample is subjected to PCR with primers
complementary to the normal target DNA wherein the
primer for extending at the 3' end of the target anneals

15   to a sequence the 3' nucleotide of which is mutated in
the mutant, the 3' primer bearing a tag or being capable
of being tagged at or on the 3' nucleotide, wherein the
presence of the tag in the PCR reaction product as
detected.

20   In this way, the absence of a detectable tag in the
product indicates that the sample does not contain
normal DNA.

An example of detecting a single base mutation is
as follows:

25   In this case, the 3' end of the primer for
extending the 3' end of the sample aligns with the
mutated DNA on the template.

Assume the case where it is to be determined
whether a patient has a normal or an abnormal genetic

30   mutation.

A normal DNA sequence of the gene in question is:

5'-ccccatg---------atgacctaggAccacct-3'
3'-ggggtac---------tactggatccTggtgga-5'

35

The patient's DNA looks like this:

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```
5'-ccccatg---------atgacctaggCccacct-3'
3'-ggggtac---------tactggatccGggtgga-5'
```

A mutation is present. The knowledge of the
patient's DNA is not necessary to do the test. Only the
normal condition needs to be known in order to diagnose
the mutation.

Primers are needed in order to do PCR, one primer
annealing in the 5'-end area, and one in 3'-end area.
The 5'-end primer will be identical for both patients
with or without the mutation, as follows:

No1-primer: 5'-ccccatg-3'

For the other 3'-area, a primer matching the normal
situation would be needed as follows:

No2normal: 5'-aggtggg-3'

If the mutation is known, a primer may be constructed
for this, as follows:

No2abnormal: 5'-aggtggt-3'

In order for the method to work, the last 3'-end
nucleotide is labelled with a biotin, still leaving it
able to elongate in a PCR reaction (having a free 3'-end
OH-group)

The 3'-end primers will now be like this:
No2normal: 5'-aggtgggB-3'
No2abnormal: 5'-aggtggtB-3'
(B=biotin)

The PCR products obtained from using these primers on
normal DNA will be:

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(No1+No2Normal primers)
```
5'-ccccatg---------atgacctaggAccacct-3'
3'-ggggtac---------tactggatccTggtgga-5'
                               |
                             biotin
```

(No1+No2abnormal primers)
```
5'-ccccatg---------atgacctaggAccacct-3'
3'-ggggtac---------tactggatccTggtgga-5'   +   biotin
```

Only use of the No2normal primer on normal template
DNA will give biotinylated PCR products. PCR enzymes
will remove the biotin from the No2abnormal primer,
giving an unbiotinylated product.

The PCR products obtained from using these primers
on abnormal DNA will be:

(No1+No2abnormal primers)
```
5'-ccccatg---------atgacctaggCccacct-3'
3'-ggggtac---------tactggatccGggtgga-5'
                               |
                             biotin
```

(No1+No2Normal primers)
```
5'-ccccatg---------atgacctaggCccacct-3'
3'-ggggtac---------tactggatccGggtgga-5'
```

As above, but just the opposite.

The presence of biotinylated product will to show
whether or not a patient has a mutation.

A specific example of diagnostic PCR of sickle cell
anaemia caused by a single base mutation is as follows:
In this example, the primer is indicated as being tagged
with biotin. This is one example of a tag which may be
used and is not to be construed as limiting this
illustrative example; other tags may be used.

Normal human hemoglobin A1 has a DNA sequence for
the first exon (coding part) of the haplotype A1 beta-
globin gene beginning as shown:

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Normal DNA:
atg gtg cac ctg act cct gag gag aag tct gcc gtt act gcc
ctg tgg ggc aag gtg aac gtg gat gaa gtt ggt ggt gag gcc
5   ctg ggc agg ...
which translates to the

Normal aminoacid sequence:
MVHLTPEEKSAVTALWGKVNVDEVGGEALG...
10

For the human disease sickle cell anaemia, the DNA
sequence for the first exon of haplotype S beta-globin
gene begins as shown:

15   Sickle cell disease DNA:
atg gtg cac ctg act cct gtg gag aag tct gcc gtt act gcc
ctg tgg ggc aag gtg aac gtg gat gaa gtt ggt ggt gag gcc
ctg ggc agg ...

20   which translates to the

Sickle cell disease aminoacid sequence:
MVHLTPVEKSAVTALWGKVNVDEVGGEALG...

25   Comparison of normal and sickle cell disease DNA
reveals that a single base substitution is sufficient to
transform the normal hemoglobin gene to an abnormal
hemoglobin gene, known to causing the serious condition
of sickle cell anaemia. The gag codon is mutated to a
30   gtg codon causing a replacement of the acidic aminoacid
glutamic acid with the nonpolar aminoacid valine.
           To detect the presence or absence of this mutation,
genomic DNA is isolated, and, if necessary, amplified by
PCR prior to diagnostic PCR in the conventional manner.
35   The primers used for amplification of part of the
hemoglobin beta chain gene are based on the DNA sequence
given by EMBEL search program when searching on the

unique identifier EMBL-ID:HSBETGLOB'.

The hemoglobin beta chain gene (shown below as separated triplets) is preceded by an intron sequence (intron 1) and followed by another sequence (intron 2), 5 both introns are indicated below in *italics*.

Part of the introns (underlined) are used for construction of primers (see below).

gcataaaagtcagggcagagccatctattgcttacatttgcttctgacacaactgt 10 *gttca ctagcaacctcaaacagacacc* atg gtg cac ctg act cct gag gag aag tct gcc gtt act gcc ctg tgg ggc aag gtg aac gtg gat gaa gtt ggt ggt gag gcc ctg ggc agg *ggcaggttggtatcaaggttac aagacaggtttaaggagaccaatagaaactgggc atgtggagacagagaagactcttgggtttctgataggcactgactctctctgccta* 15 *ttggtctattttcccacc...*

The non-coding introns are flanking the coding portion of the gene; the second intron separating the coding sequence from the next part of the coding sequence further downstream.

20 To amplify the first coding part of the gene for later diagnostic PCR, two primers may be used, for example, annealing to introns 1 and 2, respectively. Annealing sequences for the primers are indicated as *underlined italics* above.

25

Primer 1: 5' -ctagcaacctcaaacagacacc-3'
Primer 2: 5' -gtaaccttgataccaacctgcc-3'

The primers are only used in the amplifying PCR to 30 gain more DNA template for the diagnostic PCR procedure, and thus need not be biotinylated or modified in any way.

For the diagnostic PCR since the nature of the mutation is known, a single base mutation in codon 6 of 35 the exon, three primers are designed, corresponding to normal and abnormal (Sickle cell anaemia) DNA:

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Primer corresponding to Normal hemoglobin:

PrimerN: 5'-atg gtg cac ctg act cct ga-biotin-OH

Primer corresponding to the Sickle cell disease
hemoglobin:

PrimerS: 5'-atg gtg cac ctg act cct gt-biotin-OH

Primer for the other end of the gene

Primer2:   5'-gtaaccttgataccaacctgcc-3'

(This may be the same primer as used in the
amplification step; it is not tagged or modified)

Then the diagnostic test is performed with primers of
step 1 and the sample or the PCR amplified sample.

Two PCR reactions are performed

a) PCR using the PrimerN + Primer 2:

b) PCR using primerS + Primer2:

The PCR products from PCR reactions a) and b) are
then examined for presence of biotin as previously
described.

The results from examining the two PCR reactions a)
and b) can have one of four possible outcomes
illustrated below.

Biotinylated PCR product or not		Interpretation (diagnosis)
PCR # a (primerN+primer2)	PCR # b (primerS+primer2)	
Yes	Yes	Both normal and Sickle cell variants present ("harmless" heterozygous condition)
No	No	No normal gene present (unknown mutation in codon 6; may represent another disease?)
Yes	No	Only normal gene present (no mutation in codon 6; healthy subject)
No	Yes	Only mutated variant (Sickle cell) in codon 6 (possible lethal homozygous condition)

The method may also be used for diagnosis of multiple base mutations, as illustrated below, again in the case of sickle cell anaemia.

A multibase mutation (**bold underlined**) in the first exon of beta-globin gene is shown in the DNA sequence as shown:

atg gtg cac ctg act cct **aac** gag aag tct gcc gtt act gcc
ctg tgg ggc aag gtg aac gtg gat gaa gtt ggt ggt gag gcc
ctg ggc agg ...
which translates to the aminoacid sequence:
MVHLTP**N**EKSAVTALWGKVNVDEVGGEALG...

A primer such as **PrimerN** referred to above may be used to reveal whether the normal gene is present. To diagnose the potential presence of a mutation requires a primer capable of detecting the mutation.

One primer which may be used will correspond to mutated hemoglobin as follows:

5'-atggtgcacctgactcct**aac-biotin-OH**

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This primer would identify exactly the mutation
'acc' in codon 6.

Another primer which may be used is

5'-atggtgcacctgactccta-biotin-OH

5   Such a primer may be used to identify all mutations
in codon 6 starting with an 'a' base.

PCR reactions are performed with the normal
PrimerN, and with one or more primers corresponding to
(i.e. complementary to) the mutated sequence.

10   This method may be used for any mutation in a known
sequence of the normal gene and the hemoglobin Sickle
cell mutation is given as one example. Where the common
mutations seen in the population are known, a number of
primers may be used to pinpoint exactly the mutation

15   that is present.

Another utility of the method of the invention is
in excising and separating DNA fragments of interest
from a recombinant molecule for further manipulation. A
related aspect is in excising and separating vector DNA

20   for use in further cloning manipulations. There is a
need for efficient methods of obtaining high quality
linearised vector DNA fragments to be used in cloning or
other biotechnological procedures. As is known in the
art, vectors such as plasmids and viruses comprise in

25   addition to appropriate elements for controlling
replication and transcription one or more cloning sites
for incorporation of heterologous DNA for propagation or
expression. Just as restriction enzymes are used to
insert a heterologous fragment into a cloning vector to

30   prepare a recombinant vector, restriction enzymes are
also used to excise the heterologous fragment for
further genetic manipulation, or to excise the vector
element for further manipulation. Such vectors comprise
circular DNA molecules. These include a stuffer

35   fragment, often a polylinker, and a fragment which
includes the aforementioned control sequences. In one
method, a recombinant vector having an inserted

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heterologous DNA fragment is digested with a restriction
enzyme in order to excise the inserted DNA fragment
which results in a mixture of linear DNA molecules,
including the stuffer fragment of the vector itself, the
5   insert, and also partially cut recombinant DNA
molecules. The method of the invention may be used
where the stuffer (or vector) element in the recombinant
DNA molecule from which the heterologous insert is to be
excised includes a specific protein recognition
10   sequence, such as an AP-1 recognition sequence. In the
method of the invention, the products of the restriction
enzyme digestion reaction are contacted in solution with
the protein for which the nucleic acid is specific, AP-1
in this case, and then passed through a protein-
15   selective membrane which will sequester those nucleic
acid molecules to which AP-1 has bound, leaving in
solution only those DNA molecules which comprise the
heterologous inserted DNA and thus do not have an AP-1
recognition sequence.
20   In another related embodiment, the method can be
used to separate the linearised form of the vector
itself for further manipulation, ie a vector which has
been linearised by means of restriction enzyme digestion
and from which the stuffer fragment has been excised.
25   Such a vector fragment may then be used for inserting
and ligating heterologous DNA, which, following
circularisation, may be used for further downstream
applications such as for transformation of host cells.
     In one example, the stuffer fragment is or includes
30   a polylinker consisting of unique restriction enzyme
recognition sites. The vector is cut with one of these
enzymes which will result in the molecule being
linearised. The linear molecule may then be end tagged
using the aforementioned enzymatic techniques.
35   Preferably for ease of further manipulation the unique
cutting enzyme is one which creates a 3' overhang. In
this case, the linear vector molecule may be end tagged

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by the addition of tagged nucleotides such as
biotinylated nucleotides by means of TdT. If the enzyme
creates a 5' overhang, then Klenow fragment will
additionally be needed to extend the 3' termini. We
5   have found this method to be efficient, particularly
where larger quantities of DNA are involved, greater
than 10 μg. Having tagged the linearised vector, this
is then subjected to further restriction enzyme
digestion with one or more restriction enzymes
10   preferably enzymes which have unique sites within the
stuffer fragment one on either side of the original
cutting site. In this way, end fragments are tagged and
the desired fragment which is untagged may be separated
by means of the method of the invention from the stuffer
15   fragment. Thus when the sample is passed through the
filter, fully cut vector will pass through the
membrane.
        In a further application, the method of the
invention can be used to remove non-productive ligation
20   products from a ligation mixture. The covalent joining
of two DNA fragments using the enzyme DNA ligase is
central to biotechnology. In general, ligation
reactions involve insertion of small DNA fragments or
inserts into a larger vector DNA. It is important that
25   the final ligation product is correctly circularised to
avoid degradation in transformed host cells, such as
bacterial host cells. Thus linear ligation products are
of no utility and will not survive in the host bacteria.
Unfortunately, ligase reactions are not efficient,
30   resulting typically in over 80% of linear products.
These lower the efficiency of the uptake of
circularised, productive ligation products in *E. coli*.
From this point of view, the linear products can thus be
considered to be contaminants or byproducts of the
35   ligation reaction. The efficiency of transformation
with ligated products could thus be enhanced if it were
possible to remove the undesired linear ligation

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products from the reaction mixture prior to
transformation. Currently there is no method available.
Due to the polymorphic nature of the circular productive
ligation products, these cannot be recovered by excision
5   from gels. The present invention provides such a
method.

Thus according to a further aspect, this invention
provides a method of separating linear from circular
nucleic acid molecules in a sample said method
10   comprising introducing a tag to an end of a linear
nucleic acid molecule, wherein said tag is capable of
being immobilised on a matrix, by direct interaction
with the matrix or by indirect interaction by means of a
binding partner to the tag, and contacting the sample
15   with a matrix or, where the tag interacts indirectly
with the matrix, with the binding partner to the tag
and with a matrix, whereby said tagged linear nucleic
acid molecules are immobilised on the matrix.

The method of the invention is applicable in this
20   case because all DNA molecules present in the ligation
mixture other than the desired circular molecules have
free ends with exposed and reactive phosphate and
hydroxyl groups. These can be end tagged by the
aforementioned enzymatic methods with tagged nucleotides
25   such as biotinylated nucleotides. Circular molecules
cannot be tagged because they have no reactive 3'
hydroxyl groups to which a tag could be attached. In
this way, the tagged, linear molecules may be captured
by the matrix and thereby separated from the ligated
30   circularised molecules which remain in solution and can
be used for further downstream processes, such as
transformation of host cells.

In a further embodiment of the invention, the
method can be used in *in vitro* packaging of
35   bacteriophage particles, such as phage lambda, for
example in the construction of gene libraries, and phage
display libraries. Bacteriophage can be packaged either

in vitro, in which case viral DNA is mixed together with
the various virus coat protein components *in vitro*,
whereupon virus assembly occurs, or in bacteria, in
which case the viral DNA is introduced into appropriate
5   bacteria which provide the necessary protein components
needed for virus assembly. *In vitro* packaging is
quicker than the bacterial transformation method, in
that packaging can be achieved within a matter of
minutes, as opposed to 1-2 days. However, it is a less
10 efficient method than the bacterial transformation
method, particularly so in the case of packaging of
phage DNA which has been manipulated, for example λ DNA
into which heterologous DNA has been inserted, where
there are experimental indications that efficiency of
15 packaging can be reduced by as much as $10^3/\mu g$ DNA when
comparing linear DNA resulting from molecular
manipulations with unmodified linear lambda virus. This
reduced efficiency arises, at least in part, from the
presence of by products of the DNA manipulation
20 reactions. It would thus be advantageous if these by
products could be removed prior to packaging, thereby
increasing the efficiency of *in vitro* packaging and
enabling this method to be used instead of the bacterial
transformation method, allowing a speedier and thus more
25 efficient overall process. The method of the invention
makes this possible, by using the tag system to tag the
unwanted fragments, enabling them to be separated from
the desired ligation products.

The method takes advantage of the presence of cos
30 sites on the phage genome. These are single stranded
complementary regions of DNA one at each end of the
lambda genome which, upon insertion of the linear λ DNA
into bacterial cells, anneal to each other to create a
circular genome which can be replicated and which is not
35 susceptible to degradation by bacterial exonucleases as
a linear DNA would be.

In this embodiment, prior to cloning heterologous

DNA into a λ vector, the 3' ends of the λ vector are
blocked for example by adding a dideoxynucleotide
together with an appropriate DNA polymerase, for
example, klenow fragment, which effectively removes the
5   reactive OH-groups from the 3' cos sites, leaving only
an unreactive hydrogen, which is unable to be either
extended or tagged. Thus after the cloning reaction has
been carried out, the DNA molecules are subjected to the
addition of a tag capable of being added only to 3' OH
10   groups. In this way, it is only the correctly ligated
products, which do not include a reactive 3' OH group
which will not be tagged; all other DNA molecules,
including the restricted vector fragments and the
fragment to be cloned, will have a reactive 3' OH group
15   which can be tagged. Since it is only the correctly
ligated DNA molecules which cannot be tagged, these may
easily be separated from the unwanted by products using
any of the methods described herein.

       Thus viewed from a further aspect, the present
20   invention provides a method of *in vitro* phage packaging
of recombinant phage wherein the vector DNA is cut with
one or more restriction enzymes, 3' OH groups of the
vector DNA are blocked, vector and DNA to be inserted
are contacted under conditions appropriate for ligation
25   of DNA fragments, and the ligation products are tagged
with a moiety capable of attaching to reactive 3' OH
groups, followed by separation of tagged and untagged
molecules.

       In this context, blocking of the 3'OH groups means
30   that the 3'OH group is absent, or is protected or
modified in some way such that it cannot be further
extended.

       In one convenient embodiment, the tag is biotin,
which is added to the reactive 3' OH groups in the form
35   of a biotinylated nucleotide by enzymatic means, for
example by means of klenow fragment.

       In one embodiment, the 3' ends of the vector are

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first blocked, for example with a dideoxynucleotide, and
the vector then cut with an enzyme that has a single
recognition site within the vector, the recognition site
being located between the two restriction sites to be
5   used for cloning. An example of such a restriction
enzyme is EcoRI. The purpose of this initial
restriction enzyme cutting is to enable all vector DNA
molecules which are not subsequently cut to be removed
by tagging. The vector is then cut in two positions,
10 with two enzymes that have unique cutting sites within
the vector which will form the cloning site. In
addition to generating the vector fragments for cloning,
a number of partially cut restriction products may be
obtained which can be removed by means of the tag, for
15 example , where the tag is biotin, by means of
streptavidin binding, as described above. The DNA
fragment(s) for cloning are then ligated into the lambda
vector DNA. To remove all unwanted reaction products,
all exposed 3' ends are tagged, for example by means of
20 biotin. The correct ligation products do not have
reactive 3' OH groups and cannot be tagged, and may thus
be separated from all tagged molecules by the methods
described herein.

The invention will now be described in more detail
25 with reference to the following non-limiting Examples.

EXAMPLES

Example 1

5   <u>Endlabelling of restricted DNA fragments with a 5'-protruding DNA end</u>

Reagents:
   5 μg lambda HindIII restricted DNA (Gibco 15612-13)
10   40 units DNA polymerase I, Large Klenow fragment
       (New England Biolabs #210S)
   10 μl buffer for DNA polymerase I, Large Klenow fragment
       (New England Biolabs)
   5 nmol biotin-14-dCTP (Gibco 19518-018)
15   5 nmol dATP (Gibco 10216-018)
   5 nmol dTTP (Gibco 10219-012)
   5 nmol dGTP (Gibco 10218-014)
Distilled water ad 100 μl

20   The reaction mixture was incubated at 25°C for 45 minutes.

The reaction mixture was passed through a S400 HR MicroSpin column (Pharmacia-Amersham 275140-01), 10 μg
25   streptavidin (Promega #7041) was added and incubated 5 minutes at 25°C.

The reaction mixture was added to a Centriflex column (Edge Biosystems #73883) and allowed to diffuse through
30   the membrane as described by manufacturer, before centrifugation at 10000xg for 30 seconds. 10 μl distilled water was added to the membrane which was turned horizontally 180° and centrifuged again at 10000xg.
35

The eluate was analysed on 1% agarose (FMC #50080) gel electrophoresis, stained with ethidium bromide as

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described in Maniatis, Molecular Cloning: a laboratory
manual 2nd ed 1989.

No trace of lambda DNA could be seen on the gel
5   indicating that the end labelled linear molecules with
streptavidin attached were retained on the membrane.

Example 2

10   Endlabelling of a blunt-end and 3'-protruding DNA end
fragments

Reagents:

15   2 μg Low DNA Mass™ Ladder (Gibco 10068-013)
40 units Terminal Deoxynucleotidyl Transferase,
    Recombinant (Gibco 10533-016)
20 μl buffer Terminal Deoxynucleotidyl Transferase,
    Recombinant (Gibco)
20   5 nmol biotin-14-dCTP (Gibco 19518-018)
Distilled water ad 100 μl

The reaction mixture was incubated at 37°C for 45
minutes.
25

The reaction mixture was passed through a S200 HR
MicroSpin column (Pharmacia-Amersham 275120-01) and 10
μg streptavidin (Promega #7041) was added. The mixture
was incubated 5 minutes at 25°C.
30

The reaction mixture was added to a Centriflex column
(Edge Biosystems #73883) and allowed to diffuse through
the membrane as described by manufacturer, before
centrifugation at 10000xg for 30 seconds. 10 μl
35   distilled water was added to the membrane which was
turned horizontally 180° and centrifuged again at
10000xg.

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For inspection, the eluate was run on 2% agarose (FMC
#50080) gel electrophoresis, which was stained with
ethidium bromide as described in Maniatis, Molecular
Cloning: a laboratory manual 2nd ed. 1989.

No traces of DNA could be seen on the gel indicating
that the end labelled linear molecules with streptavidin
attached were retained on the membrane.

Example 3

Preparing vector DNA by removal of stuffer fragment

Cantab 5E vector containing a test insert (Pharmacia-
Amersham 279401-01) was prepared using Qiagen maxiprep
(Qiagen #12166)

Cantab 5E vector contains unique restriction sites for
the enzymes SfiI, NotI and BsmI, with the SfiI and BsmI
sites being located on either side of the NotI site

Reagents:
25 μg Cantab 5E vector as described above
40 units of NotI endonuclease (Boehringer-Mannheim
        1014714)
  5 μl buffer for NotI endonuclease (Boehringer-Mannheim
        1014714)
water ad 50 μl

The mixture was incubated 1 hour at 37°C.

The reaction mixture was passed through a S400 HR
MicroSpin column (Pharmacia-Amersham 275140-01)
whereafter the following were added:

40 units DNA polymerase I, Large Klenow fragment
        (New England Biolabs #210S)

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10 μl buffer for DNA polymerase I, Large Klenow fragment
     (New England Biolabs)
  5 nmol biotin-14-dCTP (Gibco 19518-018)
  5 nmol dGTP (Gibco 10218-014)
5 Distilled water ad 100 μl

The mixture was incubated at 25°C for 45 minutes.

The reaction mixture was passed through a S400 HR
10 MicroSpin column (Pharmacia-Amersham 275140-01).

A sample of the reaction mixture (5 μg) was diluted to
50 μl and added to a Centriflex column (Edge Biosystems
#73883) and allowed to diffuse through the membrane as
15 described by manufacturer, before centrifugation at
10000xg for 30 seconds. 10 μl distilled water was added
to the membrane which was turned horizontally 180° and
centrifuged again at 10000xg.

20 For inspection, the eluate was run on 2% agarose (FMC
#50080) gel electrophoresis, which was stained with
ethidium bromide as described in Maniatis, Molecular
Cloning: a laboratory manual 2nd ed. 1989.

25 No traces of Vector DNA were seen on the gel indicating
that all the DNA was retained on the membrane.

The reaction mixture was passed through a S400 HR
MicroSpin column (Pharmacia-Amersham 275140-01), and
30 individual aliquots treated as follows:

(1)
33 μl of the reaction mixture were placed in a separate
     tube (1) to which was added:
35 20 units BsmI (Boehringer-Mannheim 1292315)
   5 μl BsmI buffer (Boehringer-Mannheim 1292315)
distilled water ad 50 μl

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The mixture was incubated for 1 hour at 65°C.

(2)
33 µl portion of the reaction mixture were placed in a
        separate tube (2) to which was added:
20 units SfiI (Boehringer-Mannheim 1288032)
 5 µl SfiI buffer (Boehringer-Mannheim 1288032)
distilled water ad 50 µl

The mixture was incubated for 1 hour at 50°C.

(3)
33 µl portion of the reaction mixture were placed in a
        separate tube (3) to which was added:
20 units SfiI (Boehringer-Mannheim 1288032)
 5 µl SfiI buffer (Boehringer-Mannheim 1288032)
distilled water ad 50 µl

The mixture was incubated for 1 hour at 50°C.   Then the
reaction mix was passed through a S400 MicroSpin column
and the following were added to reaction mix:

20 units BsmI (Boehringer-Mannheim 1292315)
 5 µl BsmI (Boehringer-Mannheim 1292315) buffer
distilled water ad 50 µl

The mixture was incubated for 1 hour at 65°C.

Each of the three reaction mixtures were purified on
separate S400 MicroSpin columns, and 10 µg streptavidin
(Promega #7041) were added to each of the mixtures which
were incubated for 5 minutes at 25°C.

Each of the reaction mixtures were added to a Centriflex
column (Edge Biosystems #73883) and allowed to diffuse
through the membrane as described by manufacturer,
before centrifugation at 10000xg for 30 seconds.   10 µl

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distilled water was added to each membrane and the
membranes were turned horizontally 180° and centrifuged
again at 10000xg.

5   The three reaction mixtures were run on 1% agarose (FMC
#50080) gel, and stained with ethidium bromide.

Only from the third reaction sample could purified
vector DNA be seen on the gel, samples 1 and 2 gave no
10   detectable traces of DNA. This indicates that it is
only the correct restricted vector which passed through
the membrane.

Example 4

15

Restriction of biotinylated PCR fragment

A PCR product was produced amplifying a scFV construct
using high-quality biotinylated PCR primers.
20
The scFV construct is an 800 base pair fragment, which
has unique sites for SfiI and NotI, at 40 and 760 bases
respectively.

25   1 μg of PCR product was mixed with 2 μg of streptavidin
(Promega #7041) and incubated at 25°C for 5 minutes.

The reaction mix was added to a Centriflex column (Edge
Biosystems #73883) and allowed to diffuse through the
30   membrane as described by manufacturer, before
centrifugation at 10000xg for 30 seconds. 10 μl
distilled water was added to the membrane which was
turned horizontally 180° and centrifuged again at
10000xg.
35
For inspection, the reaction mix was run on 2% agarose
(FMC #50080) gel, and stained with ethidium bromide.

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No traces of the PCR product was detected, indicating
that this had been retained on the membrane.

Separate aliquots were then treated as follows:

5

(1)
  3 μg of the PCR product was added in a separate tube
     (1) to
  20 units NotI endonuclease (Boehringer-Mannheim 1014714)
10   5 μl NotI buffer and
  distilled water ad 50 μl
  and incubated for 1 hour at 37°C.

  (2)
15 Another 3 μg of the PCR product was added in a separate
     tube (2) to
  20 units SfiI (Boehringer-Mannheim 1288032)
   5 μl SfiI buffer (Boehringer-Mannheim 1288032)
  distilled water ad 50 μl
20 and incubated 1 hour at 50°C.

  (3)
  Another 3 μg of the PCR product was added in a separate
     tube (3) to
25 20 units SfiI (Boehringer-Mannheim 1288032)
   5 μl SfiI buffer (Boehringer-Mannheim 1288032)
  distilled water ad 50 μl
  and incubated 1 hour at 50°C.

30 Then the reaction was passed through a S400 MicroSpin
  column and the following were added to the reaction mix:

  20 units NotI endonuclease (Boehringer-Mannheim 1014714)
   5 μl NotI endonuclease Boehringer-Mannheim 1014714)
35 buffer
  distilled water ad 50 μl
  and incubated for 1 hour at 37°C.

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Each of the three reaction mixtures were purified on separate S400 MicroSpin columns, and 10 μg streptavidin (Promega #7041) were subsequently added to each of the mixtures which were incubated for 5 minutes at 25°C.

5

Each of the reaction mixtures were added to a Centriflex column (Edge Biosystems #73883) and allowed to diffuse through the membrane as described by manufacturer, before centrifugation at 10000xg for 30 seconds. 10 μl

10   distilled water was added to each membrane, and the membranes were turned horizontally 180° and centrifuged again at 10000xg.

For inspection, the three reaction mixtures were run on

15   1% agarose (FMC #50080) gel, and stained with ethidium bromide.

Only from the reaction mix in tube 3 could purified PCR product be seen on the gel, tubes 1 and 2 gave no

20   detectable traces of DNA, indicating that end-containing fragments had been retained on the membrane, and the internal fragment had passed through the membrane.

Example 5

25

Restriction of non-biotinylated PCR fragment

A PCR product was produced amplifying a scFV construct using high-quality biotinylated PCR primers.

30

1 μg of PCR product was mixed with 2 μg of streptavidin (Promega #7041) and incubated at 25°C for 5 minutes, as per Example 4.

35   The reaction mix was added to a Centriflex column (Edge Biosystems #73883) and allowed to diffuse through the membrane as described by manufacturer, before

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centrifugation at 10000xg for 30 seconds. 10 µl
distilled water was added to the membrane which was
turned horizontally 180° and centrifuged again at
10000xg.

For inspection, the reaction mix was run on 2% agarose
(FMC #50080) gel, and stained with ethidium bromide.

The PCR product was detected on the gel, indicating that
it had not been retained on the membrane.

The following were added to 3 µg PCR product:
40 units Terminal Deoxynucleotidyl Transferase,
    Recombinant (Gibco 10533-016)
20 µl buffer Terminal Deoxynucleotidyl Transferase,
    Recombinant (Gibco)
 5 nmol biotin-14-dCTP (Gibco 19518-018)
Distilled water ad 100 µl
and incubated at 37°C for 45 minutes.

The reaction mixture was passed through a S200 HR
MicroSpin column (Pharmacia-Amersham 275120-01), 10 µg
streptavidin (Promega #7041) was added and incubated 5
minutes at 25°C.

The reaction mixture was added to a Centriflex column
(Edge Biosystems #73883) and allowed to diffuse through
the membrane as described by manufacturer, before
centrifugration at 10000xg for 30 seconds. 10 µl
distilled water was added, the column turned
horizontally 180° and centrifuged again at 10000xg.

For inspection, the reaction mixture was run on 2%
agarose (FMC #50080) gel, and stained with ethidium
bromide.

No PCR product could be detected on gel, indicating that

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it had been retained on the membrane.

Three separate aliquots were treated as follows:

5   (1)
        3 μg of the PCR product was added in a separate tube
          (1) to
        20 units NotI endonuclease (Boehringer-Mannheim 1014714)
         5 μl NotI buffer and
10   distilled water ad 50 μl
        and incubated for 1 hour at 37°C.

        (2)
        Another 3 μg of the PCR product was added in a separate
15   tube (2) to
        20 units SfiI (Boehringer-Mannheim 1288032)
         5 μl SfiI buffer (Boehringer-Mannheim 1288032)
        distilled water ad 50 μl
        and incubated 1 hour at 50°C.
20
        (3)
        Another 3 μg of the PCR product was added in a separate
            tube (3) to
        20 units SfiI (Boehringer-Mannheim 1288032)
25   5 μl SfiI buffer (Boehringer-Mannheim 1288032)
        distilled water ad 50 μl
        and incubated 1 hour at 50°C.

        Then the reaction was passed through a S400 MicroSpin
30   column and the following were added to the reaction mix:

        20 units NotI endonuclease (Boehringer-Mannheim 1014714)
         5 μl NotI endonuclease (Boehringer-Mannheim 1014714)
        buffer
35   distilled water ad 50 μl
        and incubated for 1 hour at 37°C.

Each of the three reaction mixtures were purified on
separate S400 MicroSpin columns, and 10 µg streptavidin
(Promega #7041) subsequently added and incubated for 5
minutes at 25°C.

Each of the three reaction mixtures were added to a
Centriflex column (Edge Biosystems #73883) and allowed
to diffuse through the membrane as described by
manufacturer, before centrifugation at 10000xg for 30
seconds. 10 µl distilled water was added to each
membrane and the membranes were turned horizontally 180°
and centrifuged again at 10000xg.

For inspection, the three reaction mixtures were run on
1% agarose (FMC #50080) gel, and stained with ethidium
bromide.

Only from the reaction mix group 3 could purified PCR
product be seen on the gel, tubes 1 and 2 gave no
detectable traces of DNA, indicating that end containing
fragments had been retained on the membrane, and the
internal fragment had passed through the membrane.

Example 6

Removal of linear DNA from a population of circular and
linear DNA molecules

The circular DNA starting material is the cloning vector
pUC19 which has a unique HindIII site in the polylinker
cloning site.

    5 µg pUC19 vector DNA (New England Biolabs #301-1S) was
        added to:
40 units of HindIII restriction enzyme (New England
        Biolabs #104S)
    5 µl buffer for HindIII restriction enzyme (New England

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Biolabs)
distilled water ad 50 μl

Incubated 1 hour at 37°C.

5

The reaction was passed through a S400 HR MicroSpin
column and the reaction mix added to:

5 μl pUC19 vector DNA (New England Biolabs #301-1S)
10   40 units DNA polymerase I, Large Klenow fragment
            (New England Biolabs #210S)
10 μl buffer for DNA polymerase I, Large Klenow fragment
            (New England Biolabs)
  5 nmol biotin-14-dCTP (Gibco 19518-018)
15   5 nmol dATP (Gibco 10216-018)
  5 nmol dTTP (Gibco 10219-012)
  5 nmol dGTP (Gibco 10218-014)
Distilled water ad 100 μl

20   The reaction mixture was incubated at 25°C for 45
minutes.

The reaction mixture was passed through a S400 HR
MicroSpin column (Pharmacia-Amersham 275140-01), 10 μg
25   streptavidin (Promega #7041) was added and incubated 5
minutes at 25°C.

The reaction mixture was added to a Centriflex column
(Edge Biosystems #73883) and allowed to diffuse through
30   the membrane as described by manufacturer, before
centrifugration at 10000xg for 30 seconds. 10 μl
distilled water was added, the column turned
horizontally 180° and centrifuged again at 10000xg.

35   For inspection, the eluate was analysed on 1% agarose
(FMC #50080) gel electrophoresis, stained with ethidium
bromide as described in Maniatis, Molecular Cloning: a

laboratory manual 2nd ed 1989.

Only circular vector was detected on the agarose (FMC
#50080) gel, indicating that it had passed through the
5   membrane, the linear DNA having been retained.

Example 7

In vitro packaging of lambda phage, using precut lambda
10   *vector*

The precut lambda vector Uni-ZAP® XR (Stratagene, cat.
#236612) is used.

15   Reagents:
     10 μg precut lambda vector
     dideoxyguanosine triphosphate (200 μM final
     concentration)
     dideoxyadenosine triphosphate (200 μM final
20   concentration)
     Klenow fragment (10 units)
     NEB EcoPol buffer
     in a total volume of 30 μl

25   The reaction mixture is incubated at 24°C for 20
     minutes.

     The reaction mixture is passed through a S400 HR
     MicroSpin column to remove proteins and nucleotides and
30   to the purified reaction mix is added:

     200 ng of pre-prepared DNA insert
     T4 DNA ligase
     NEB T4 ligase buffer
35   in a volume of 40 μl

     The reaction mixture is incubated at 16°C for 48 hours.

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The reaction mixture is passed through a S400 HR
MicroSpin column and to the purified reaction mix is
added:
Biotinylated dCTP (5 nmol)
5   Biotinylated dATP (5 nmol)
dTTP (200 μM final concentration)
dGTP (200 μM final concentration)
Klenow fragment (10 units)
NEB buffer
10   in a volume of 50 μl

The reaction mixture is incubated at 24^{0}C for 20 minutes.

The reaction mixture is passed through a S400 HR
15   MicroSpin column and to the purified reaction mix is
added Streptavidin and the reaction mixture added to a
Centriflex column as in Example 2.

Further processing follows the instructions of the
20   packaging insert of Stratagene. The ligation gives a
higher number of viable bacteriophages than found in the
standard method, as seen by plaque count on bacteria.

Example 8

25

*In vitro packing of lambda phage using uncut lambda
vector*

The uncut lambda vector Uni-ZAP® XR (Stratagene)
30   containing a test insert is used.

Reagents:
5 μg uncut lambda vector
dideoxyguanosine triphosphate (200 μM final
35   concentration)
dideoxyadenosine triphosphate (200 μM final
concentration)

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Klenow fragment (10 units)
NEB buffer
in a total volume of 20 μl

5   The reaction mixture is incubated at 24°C for 20 minutes.
This blocks the 3' ends of the vector.

The reaction mixture is passed through a S400 HR
MicroSpin column and to the purified reaction mix is
10   added:

EcoRI restriction enzyme (20 units)
NEB EcoRI buffer
in a volume of 30 μl.
15

The reaction mixture is incubated at 37°C for 120
minutes. This digests the vector into approximately
equal pieces.

20   The reaction is passed through a S400 HR MicroSpin
column and to the purified reaction mix is added:

Biotinylated dATP (5 nmol)
dTTP (200 μM final concentration)
25   dCTP (200 μM final concentration)
dGTP (200 μM final concentration)
Klenow fragment (10 units)
NEB buffer
in a volume of 40 μl
30

The reaction mixture is incubated at 24°C for 30 minutes.
In this reaction, biotin will label the EcoRI digested
lambda

35   To the reaction mixture is added:

Xbal and Xhol restriction enzymes (20 units of each) in

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NEB 2 buffer in a volume of 50 μl. This reaction
creates the cloning site.

The reaction mixture is incubated at 37°C for 60
5   minutes.

The reaction is passed through a S400 HR MicroSpin
column and to the purified reaction mix is added
Streptavidin and the reaction mixture added to a
10   Centriflex column as in example 2. In this step,
Streptavidin binds to uncut vector.

The reaction is passed through a S400 HR MicroSpin
column and to the purified reaction mix is added:
15

200 ng of pre-prepared DNA insert
T4 DNA ligase
NEB T4 ligase buffer
in a volume of 60 μl
20

The reaction mixture is incubated at 16°C for 48 hours.

The reaction mixture is passed through a S400 HR
MicroSpin column and to the purified reaction mix is
25   added:

Biotinylated dCTP (5 nmol)
Biotinylated dATP (5 nmol)
dTTP (200 μM final concentration)
30   dGTP (200 μM final concentration)
Klenow fragment (10 units)
NEB buffer
in a volume of 70 μl

35   The reaction mixture is incubated at 24°C for 20 minutes.
In this reaction, all exposed 3' OH ends not previously
blocked are labelled.

The reaction mixture is passed through a S400 HR
MicroSpin column and to the purified reaction mix is
added Streptavidin and the reaction mixture added to a
Centriflex column as in example 2. This separates the
tagged from the untagged molecules.

Further processing follows the instructions of the
packaging insert of Stratagene. The ligation gives a
higher number of viable bacteriophages than found in the
standard method, as seen by plaque count on bacteria.

Example 9

Clean-up of reamplification PCR reaction

a)   Modification of Cantab5E vector (Pharmacia) to
obtain Cantab5E$^{BamHI(1)}$

Cantab5E vector (1 μg) is cut by adding 10 units of
BamHI and 10 μl NEB BamHI buffer in of total reaction
volume of 100 μl and incubated for 1 hour at 37°C. The
reaction mixture is separated on 1% agarose gel and the
fragment of highest molecular weight is isolated by
common gel extraction method. To 500 ng of the isolated
large fragment is added 1 Weiss unit of T4 DNA ligase
and 5 μl NEB ligase buffer in a total reaction volume of
50 μl and the mixture is incubated for 1 hour at 24°C.
The ligation mixture is transformed into TGI cells using
electroporation and the bacterial cells are incubated
over night on ampicillin-containing agar (100 μg/ml).
DNA from colony-forming bacteria is isolated and
verified by size and restriction enzymes to contain only
one BamHI restriction site. This DNA is named Cantab5E
$^{BamHI(1)}$.

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b)   Using the modified vector Cantab5E$^{BamHI(1)}$ as a template in PCR

Reagents:

5   10 μg Cantab5E$^{BamHI(1)}$ vector

dNTP mix (supplied by Finnzyme; 200 μM final concentration)

10 μl expand high fidelity buffer (Boehringer-Mannheim AG)

10   30 pmol primer 1 that is partly overlapping with a unique afiIII restriction site 30 pmol primer 2 that is partly overlapping with a unique BamHI restriction site water ad 100 μl

The PCR reaction was heated to 96^{0}C for 2 minutes before

15   adding 1 μl expand high fidelity enzyme (Boehringer-Mannheim AG).

The reaction mixture is cycled on a thermo-cycler for 20 rounds, at following conditions:

20

-    First 2 minutes at 96^{0}C, then 20 rounds as follows
-    30 seconds at 55^{0}C,
-    80 seconds at 72^{0}C,
-    30 seconds at 96^{0}C.

25

The PCR product is identified by ethidiumbromide staining after electrophoresis on agarose. Typically unwanted by-products are observed.

30   The thermo-cycled reaction mixture is passed through a S400 HR MicroSpin column.

The purified PCR product is cut using restriction enzymes AflIII and BamHI in a normal procedure, followed

35   by adding streptavidin, and the reaction mixture added to a Centriflex column (as in example 2). Only correctly cut PCR product is seen upon Agarose

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electrophoresis, as unwanted impurities are retained by
the protein-binding matrix.

Example 10

Use of biotin-containing DNA in diagnostic PCR of sickle
cell anaemia

Normal human hemoglobin A1 has a DNA sequence for the
first exon (coding part) of the haplotype A1 beta-globin
gene beginning as shown:

Normal DNA:
atg gtg cac ctg act cct gag gag aag tct gcc gtt act gcc
ctg tgg ggc aag gtg aac gtg gat gaa gtt ggt ggt gag gcc
ctg ggc agg ...
which translates to the

Normal aminoacid sequence:
MVHLTPEEKSAVTALWGKVNVDEVGGEALG...

For the human disease sickle cell anaemia, the DNA
sequence for the first exon of haplotype S beta-globin
gene begins as shown:

Sickle cell disease DNA:
atg gtg cac ctg act cct gtg gag aag tct gcc gtt act gcc
ctg tgg ggc aag gtg aac gtg gat gaa gtt ggt ggt gag gcc
ctg ggc agg ...

which translates to the

Sickle cell disease aminoacid sequence:
MVHLTPVEKSAVTALWGKVNVDEVGGEALG...

Comparison of normal and sickle cell disease DNA reveals
that a single base substitution is sufficient to

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transform the normal hemoglobin gene to an abnormal
hemoglobin gene, known to causing the serious condition
of sickle cell anaemia. The gag codon is mutated to a
gtg codon causing a replacement of the acidic aminoacid
5   glutamic acid with the nonpolar aminoacid valine.

To detect the presence or absence of this mutation,
genomic DNA is isolated, and, if necessary, amplified by
PCR prior to diagnostic PCR in the conventional manner.
10

Example 10(a) Isolation of patient DNA

A commercial kit (GFX Genomic Blood DNA Purification
kit; Pharmacia-Amersham #27-9603-01) is used according
15   to manufacturers recommendation to isolate genomic DNA
from a patient blood sample.

If sufficient DNA is obtained, one proceeds directly to
Example 10c. If not, a PCR amplification step of part
20   of the hemoglobin beta chain gene is performed (Example
10b).

Example 10(b) Amplifying step (optional, if needed for
lack of material)
25

The isolated DNA of Example 10a is amplified to increase
the amount of DNA for further processing, as shown
below.

30   A mixture of

250 ng genomic DNA
10 μl high expand fidelity buffer
20 nmol dNTP
35   20 pmol of each amplification primer (Primer 1 and
Primer 2; see below)
Water to 100 μl

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3 units of Expand High Fidelity PCR enzyme

is used for PCR for 30 rounds at the following standard
conditions:

5

hotstart at 96°C
annealing at 56° for 30 seconds
polymerization at 72°C for 1 minute
denaturation 96°C for 30 seconds

10

The primers used for amplification of part of the
hemoglobin beta chain gene are based on the DNA sequence
given by EMBEL search program when searching on the
unique identifier EMBL-ID:HSBETGLOB'.

15

The hemoglobin beta chain gene (shown below as separated
triplets) is preceded by an intron sequence (intron 1)
and followed by another sequence (intron 2), both
introns are indicated below in *italics*.

20

Part of the introns (<u>underlined</u>) are used for
construction of primers (see below).

gcataaaagtcagggcagagccatctattgcttacatttgcttctgacacaactgt
25   *gttca<u>ctagcaacctcaaacagacacc</u>* atg gtg cac ctg act cct gag
gag aag tct gcc gtt act gcc ctg tgg ggc aag gtg aac gtg
gat gaa gtt ggt ggt gag gcc ctg ggc agg
<u>ggcaggttggtatcaaggttac</u>aagacaggtttaaggagaccaatagaaactgggc
atgtggagacagagaagactcttgggtttctgataggcactgactctctctgccta
30   *ttggtctattttcccacc...*

The non-coding introns are flanking the coding portion
of the gene; the second intron separating the coding
sequence from the next part of the coding sequence
35   further downstream.

To amplify the first coding part of the gene for later

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diagnostic PCR, two primers may be used, for example, annealing to introns 1 and 2, respectively. Annealing sequences for the primers are indicated as _underlined italics_ above.

5

Primer 1: 5' -ctagcaacctcaaacagacacc-3'
Primer 2: 5' -gtaaccttgataccaacctgcc-3'

10   The primers are only used in the amplifying PCR to gain more DNA template for the diagnostic PCR procedure, and thus need not be biotinylated or modified in any way.

Example 10c   Diagnostic PCR

15

Step 1 - construction of primers

For the diagnostic PCR since the nature of the mutation is known, a single base mutation in codon 6 of the exon,
20   three primers are designed, corresponding to normal and abnormal (Sickle cell anaemia) DNA:

Primer corresponding to Normal hemoglobin:
    PrimerN: 5'-atg gtg cac ctg act cct ga-biotin-OH

25

Primer corresponding to the Sickle cell disease hemoglobin:
    PrimerS: 5'-atg gtg cac ctg act cct gt-biotin-OH

30   Primer for the other end of the gene
    Primer2:   5'-gtaaccttgataccaacctgcc-3'
(This may be the same primer as used in the amplification step of Example 10b; it is not tagged or modified).

35

Step 2 - diagnostic test with primers of step 1 and the PCR product from Example 10b

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Two PCR reactions are performed, using the same
conditions as in example 10b.

5   a) PCR using the PrimerN + Primer 2:

100 ng PCR product DNA from Example 10b
10 µl high expand fidelity buffer
20 nmol dNTP
10   20 pmol of each amplification primer (Primer N and
Primer2)
3 units of Expand High Fidelity PCR enzyme
water to 100 µl

15   b) PCR using primerS + Primer2:
as for PCR reaction (a) but with amplification primer AB
replacing primer N

The PCR products from PCR reactions a) and b) are
20   purified by the use of S400HR MicroSpin columns as
described in Example 1. The products are then examined
for presence of biotin by means of a centriflex membrane
as described in Example 1. The reaction mixture is
divided into two portions. Excess streptavidin (5 µg)
25   is added to 9/10 of the reaction mixtures and incubated
at 25°C for 5 minutes as in Example 1 then added to a
centriflex membrane as in Example 1. The collected
eluate is analysed on an agarose gel, as in Example 1,
for the presence of a DNA product. The remaining 1/10 of
30   the reaction mixture which has not been contacted with
streptavidin is run in parallel on the agarose gel as a
control sample.

The results from examining the two PCR reactions a) and
35   b) can have one of four possible outcomes illustrated
below.

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Biotinylated PCR product or not		Interpretation (diagnosis)
PCR # a (primerN+primer2)	PCR # b (primerS+primer2)	
Yes	Yes	Both normal and Sickle cell variants present ("harmless" heterozygous condition)
No	No	No normal gene present (unknown mutation in codon 6; may represent another disease?)
Yes	No	Only normal gene present (no mutation in codon 6; healthy subject)
No	Yes	Only mutated variant (Sickle cell)in codon 6 (possible lethal homozygous condition)

The method may also be used for diagnosis of multiple base mutations, as illustrated below, again in the case of sickle cell anaemia.

Example 11

Diagnostic PCR of sickle cell anaemia (multiple base mutation)

A multibase mutation (**bold underlined**)in the first exon of beta-globin gene is shown in the DNA sequence as shown:

atg gtg cac ctg act cct **aac** gag aag tct gcc gtt act gcc
ctg tgg ggc aag gtg aac gtg gat gaa gtt ggt ggt gag gcc
ctg ggc agg ...

which translates to the aminoacid sequence:
MVHLTP**N**EKSAVTALWGKVNVDEVGGEALG...

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Although three bases have changed, a similar method as
in Example 10 can be used to detect the mutation

As in Example 10, a **PrimerN** is used to reveal whether
5   the normal gene is present. Two primers are constructed
to diagnose the potential presence of a mutation.

One primer which corresponds to mutated hemoglobin as
follows:
10 Primer AB2a
        5'-atggtgcacctgactcct<u>aac-biotin-OH</u>

This primer identifies exactly the mutation '<u>acc</u>' in
codon 6.
15
The second primer is
Primer AB2b
        5'-atggtgcacctgactcct<u>a-biotin-OH</u>

20 This primer may identify all mutations in codon 6
starting with an '<u>a</u>' base.

PCR reactions are performed with the normal PrimerN, and
with one or more primers corresponding to (i.e.
25 complementary to) the mutated sequence using the
conditions of Example 10.

This method may be used for any mutation in a known
sequence of the normal gene and the hemoglobin Sickle
30 cell mutation is given as one example. Where the common
mutations seen in the population are known, a number of
primers may be used to pinpoint exactly the mutation
that is present.

35 <u>Examples 12-22</u>

The Examples 1-11 are repeated, with a NycoCard bearing

a protein binding membrane replacing the centriflex
column for removal or isolation of biotin-labelled and
steptavidin-complexed DNA molecules.

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Claims

1.   A method for at least partially separating nucleic
acid molecules in a sample into populations wherein a
population is tagged or capable of being tagged with a
moiety capable of being immobilised on a matrix, said
method comprising contacting the nucleic acid containing
sample with a matrix whereby the tagged molecules are
captured by the matrix and thereby separated from
untagged molecules.

2.   A method according to claim 1 wherein a population
of molecules is tagged or capable of being tagged with a
moiety capable of being immobilised on a matrix either
directly or indirectly via a binding partner for the
tag, said method comprising contacting the nucleic acid
containing sample with a matrix, or, where the tag
interacts indirectly with the matrix by means of a
binding partner, with a binding partner for the tag and
with a matrix, whereby tagged molecules are captured by
the matrix and thereby separated from untagged
molecules.

3.   A method as claimed in any one of the preceding
claims wherein the tag is a moiety which can be
incorporated into a nucleic acid molecule or a moiety
which has an affinity for a nucleic acid molecule.

4.   A method as claimed in any one of the preceding
claims wherein the tag interacts directly with the
matrix.

5.   A method as claimed in any one of claims 1 to 3
wherein the tag interacts indirectly with the matrix by
means of a binding partner for the tag.

6.   A method as claimed in any one of the preceding

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claims wherein the tag is a ligand.

7.   A method as claimed in claim 6 wherein the ligand
is selected from biotin or fluorescein.

8.   A method as claimed in any one of claims 1 to 5
wherein the tag is a steroid or a steroid like molecule.

9.   A method as claimed in claim 8 wherein the steroid
is digoxygenin.

10.   A method as claimed in any one of claims 1 to 5
wherein the tag is an antigen.

11.   A method as claimed in any one of claims 1 to 5
wherein the tag is a protein.

12.   A method as claimed in claim 11 wherein the protein
is a nucleic acid binding protein.

13.   A method as claimed in any one of claims 1 to 5
wherein the tag is a nucleic acid molecule.

14.   A method as claimed in any one of claims 1 to 7
wherein the tag is biotin, and the tag is captured by
means of streptavidin or avidin.

15.   A method as claimed in any one of the preceding
claims wherein the matrix is in the form of sheets,
gels, filters, membranes, fibres, tubes, microtitre
plates, columns, particles.

16.   A method as claimed in claim 15 wherein the matrix
is a porous material.

17.   A method as claimed in claim 16 wherein the matrix
is incorporated into a separation device selected from

centrifuge vials, microtiter plates, cartridges and
syringes.

18.   A method as claimed in any one of claims 15 to 17
wherein an absorbent pad is located on said porous
material, a liquid impermeable sheet is located on the
face of said absorbent pad remote from said porous
material, and a liquid impermeable sheet having one or
more holes therein is located on the face of said porous
material remote from said absorbent pad, whereby the
test sample is applied to one of said holes and is
caused to diffuse transversely through said porous
material by absorption into said absorbent pad.

19.   A method as claimed in any one of the preceding
claims for the at least partial separation of a product
of restriction enzyme digestion, or for purifying the
products of the PCR reaction.

20.   A method as claimed in any one of the preceding
claims for at least partially separating a mixture of
restriction enzyme digested fragments of DNA wherein the
starting material is a linear DNA molecule which is
tagged or capable of being tagged at or near one or both
ends with a moiety capable of being immobilised on a
matrix, said method comprising subjecting the DNA
molecule to restriction enzyme digestion followed by
contacting the sample with a matrix whereby the tagged
molecules which originate from an end of the starting
material are captured by the matrix and are thereby
separated from untagged molecules.

21.   A method as claimed in any one of the preceding
claims for at least partially separating the correct and
desired products of PCR amplification from PCR products
which result from incorrect annealing of a PCR primer to
template, wherein the template nucleic acid molecule

comprises a unique restriction enzyme recognition site
at or towards an end of the template, and a PCR primer
which is tagged or capable of being tagged is
complementary to a sequence on the template which
extends partially into the unique restriction site, the
method comprising amplifying the template by means of
PCR, digesting the PCR products with the restriction
enzyme specific for the said unique restriction enzyme
recognition site, and contacting the resulting product
with a matrix capable of sequestering the tag whereby
tagged nucleic acid molecules are captured by the matrix
and thereby separated from untagged molecules.

22.   A method as claimed in claim 21 wherein the tag is
attached at the 5' end of the primer.

23.   A method as claimed in any one of claims 1 to 18
for diagnostic PCR.

24.   A method as claimed in claim 23 of diagnostic PCR
wherein a test sample is separately subjected to PCR
reactions, in which the mutation, if present, is in the
sequence to which the 3' primer is complementary, a
first PCR reaction using a 3' primer complementary to
the normal target nucleic acid and a second PCR reaction
using a 3' primer complementary to the mutant target,
wherein the 3' nucleotide of the mutant primer
corresponds to one of the nucleotides which is mutated
in the mutant nucleic acid, each of said 3' primers
bearing a tag or being capable of being tagged on the 3'
nucleotide of the primer, wherein the presence or
absence of the tag in the PCR reaction products is
detected.

25.   A method as claimed in claim 23 of diagnostic PCR
of mutations in a nucleic acid molecule wherein the
presence or absence of a mutation in a nucleic acid

sample is detected, wherein a 3' primer specific for
either the normal or mutant nucleic acid is used,
wherein said primer is complementary to a region of the
nucleic acid where there is a base difference between
5   the normal and mutated DNA with the 3' terminus of the
primer corresponding to the position in the sample where
there is a difference between the normal and mutant, the
primer being tagged or capable of being tagged, whereby
the presence or absence of the tag in the PCR product is
10   detected.

26. A method as claimed in claim 23 of diagnostic PCR
in which a test sample is subjected to PCR with primers
complementary to the normal target DNA wherein the
15   primer for extending at the 3' end of the target anneals
to a sequence the 3' nucleotide of which is mutated in
the mutant, the 3' primer bearing a tag or being capable
of being tagged at or on the 3' nucleotide, wherein the
presence of the tag in the PCR reaction product as
20   detected.

27. A method as claimed in any one of claims 1 to 18
for separating linear from circular DNA molecules.

25   28. A method as claimed in claim 27 of separating
linear from circular nucleic acid molecules in a sample
said method comprising introducing a tag to an end of a
linear nucleic acid molecule, wherein said tag is
capable of being immobilised on a matrix, by direct
30   interaction with the matrix or by indirect interaction
by means of a binding partner to the tag, and
contacting the sample with a matrix or, where the tag
interacts indirectly with the matrix, with the binding
partner to the tag and with a matrix, whereby said
35   tagged linear nucleic acid molecules are immobilised on
the matrix.

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29.   A method as claimed in any one of claims 1 to 18 for *in vitro* packaging of bacteriophage particles.

30.   A method as claimed in claim 29 of *in vitro* phage
5   packaging of recombinant phage wherein the vector DNA is
      cut with one or more restriction enzymes, 3' OH groups
      of the vector DNA are blocked, vector and DNA to be
      inserted are contacted under conditions appropriate for
      ligation of DNA fragments, and the ligation products are
10   tagged with a moiety capable of attaching to reactive 3'
      OH groups, followed by separation of tagged and untagged
      molecules.

31.   A method for at least partially separating nucleic
15   acid molecules in a sample into populations wherein a
      population is tagged or capable of being tagged with a
      moiety capable of being immobilised on a matrix either
      directly or indirectly via a binding partner for the
      tag, said method comprising contacting the nucleic acid
20   containing sample with a matrix, or, where the tag
      interacts indirectly with the matrix by means of a
      binding partner, with a binding partner for the tag and
      with a matrix, whereby tagged molecules are captured by
      the matrix and thereby separated from untagged
25   molecules.

32.   A method for at least partially separating a
      mixture of restriction enzyme digested fragments of DNA
      wherein the starting material is a linear DNA molecule
30   which is tagged or capable of being tagged at or near
      one or both ends with a moiety capable of being
      immobilised on a matrix, said method comprising
      subjecting the DNA molecule to restriction enzyme
      digestion followed by contacting the sample with a
35   matrix whereby the tagged molecules which originate from
      an end of the starting material are captured by the
      matrix and are thereby separated from untagged

molecules.

33.   A method for at least partially separating the
correct and desired products of PCR amplification from
PCR products which result from incorrect annealing of a
PCR primer to template, wherein the template nucleic
acid molecule comprises a unique restriction enzyme
recognition site at or towards an end of the template,
and a PCR primer which is tagged or capable of being
tagged is complementary to a sequence on the template
which extends partially into the unique restriction
site, the method comprising amplifying the template by
means of PCR, digesting the PCR products with the
restriction enzyme specific for the said unique
restriction enzyme recognition site, and contacting the
resulting product with a matrix capable of sequestering
the tag whereby tagged nucleic acid molecules are
captured by the matrix and thereby separated from
untagged molecules.

34.   A method of diagnostic PCR wherein a test sample is
separately subjected to PCR reactions, in which the
mutation, if present, is in the sequence to which the 3'
primer is complementary, a first PCR reaction using a
3' primer complementary to the normal target nucleic
acid and a second PCR reaction using a 3' primer
complementary to the mutant target, wherein the 3'
nucleotide of the mutant primer corresponds to one of
the nucleotides which is mutated in the mutant nucleic
acid, each of said 3' primers bearing a tag or being
capable of being tagged on the 3' nucleotide of the
primer, wherein the presence or absence of the tag in
the PCR reaction products is detected.

35.   A method of diagnostic PCR of mutations in a
nucleic acid molecule wherein the presence or absence of
a mutation in a nucleic acid sample is detected, wherein

a 3' primer specific for either the normal or mutant
nucleic acid is used, wherein said primer is
complementary to a region of the nucleic acid where
there is a base difference between the normal and
5   mutated DNA with the 3' terminus of the primer
corresponding to the position in the sample where there
is a difference between the normal and mutant, the
primer being tagged or capable of being tagged, whereby
the presence or absence of the tag in the PCR product is
10   detected.

36.   A method of diagnostic PCR in which a test sample
is subjected to PCR with primers complementary to the
normal target DNA wherein the primer for extending at
15   the 3' end of the target anneals to a sequence the 3'
nucleotide of which is mutated in the mutant, the 3'
primer bearing a tag or being capable of being tagged at
or on the 3' nucleotide, wherein the presence of the tag
in the PCR reaction product as detected.

20

37.   A method of separating linear from circular nucleic
acid molecules in a sample said method comprising
introducing a tag to an end of a linear nucleic acid
molecule, wherein said tag is capable of being
25   immobilised on a matrix, by direct interaction with the
matrix or by indirect interaction by means of a binding
partner to the tag, and contacting the sample with a
matrix or, where the tag interacts indirectly with the
matrix, with the binding partner to the tag and with a
30   matrix, whereby said tagged linear nucleic acid
molecules are immobilised on the matrix.

38.   A method of *in vitro* phage packaging of recombinant
phage wherein the vector DNA is cut with one or more
35   restriction enzymes, 3' OH groups of the vector DNA are
blocked, vector and DNA to be inserted are contacted
under conditions appropriate for ligation of DNA

fragments, and the ligation products are tagged with a
moiety capable of attaching to reactive 3' OH groups,
followed by separation of tagged and untagged molecules.

INTERNATIONAL SEARCH REPORT

A. CLASSIFICATION OF SUBJECT MATTER

IPC 7   C12Q1/68   C12N15/10

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7   C12Q   C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 90 12115 A (COLLABORATIVE RES INC) 18 October 1990 (1990-10-18) page 24-31; claims; example 1	1-20, 23-26, 31,36
X	EP 0 872 559 A (SHIONOGI & CO) 21 October 1998 (1998-10-21) the whole document	1-20,31, 32
X	US 5 602 300 A (GOSSEN JAN A ET AL) 11 February 1997 (1997-02-11) the whole document	1-20,31
X	WO 85 04674 A (LIFE TECHNOLOGIES INC) 24 October 1985 (1985-10-24) page 5-7	1,15-20

-/—

[X] Further documents are listed in the continuation of box C.   [X] Patent family members are listed in annex.

* Special categories of cited documents :

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
7 April 2000	13/04/2000

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31–70) 340–2040, Tx. 31 651 epo nl, Fax: (+31–70) 340–3016	Reuter, U

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
A	WO 97 28282 A (STRATAGENE INC) 7 August 1997 (1997-08-07) the whole document	1-38
A	FR 2 678 639 A (RHONE POULENC RORER SA) 8 January 1993 (1993-01-08) the whole document	1-38
A	US 5 741 678 A (RONAI ZEEV A) 21 April 1998 (1998-04-21) the whole document	1,21,33

3

Form PCT/ISA/210 (continuation of second sheet) (July 1992)

Patent document cited in search report		Publication date	Patent family member(s)		Publication date
WO 9012115	A	18-10-1990	AU	5358990 A	22-10-1990
			AU	5640090 A	05-11-1990
			WO	9011372 A	04-10-1990
			US	5391480 A	21-02-1995
EP 0872559	A	21-10-1998	AU	7506896 A	29-05-1997
			WO	9717466 A	15-05-1997
US 5602300	A	11-02-1997	NL	9100567 A	02-11-1992
			AT	128735 T	15-10-1995
			DE	69205277 D	09-11-1995
			DE	69205277 T	21-03-1996
			DK	579713 T	04-12-1995
			EP	0579713 A	26-01-1994
			ES	2078043 T	01-12-1995
			GR	3017795 T	31-01-1996
			JP	6506357 T	21-07-1994
			WO	9217605 A	15-10-1992
WO 8504674	A	24-10-1985	EP	0177497 A	16-04-1986
			JP	61501746 T	21-08-1986
WO 9728282	A	07-08-1997	NONE		
FR 2678639	A	08-01-1993	NONE		
US 5741678	A	21-04-1998	US	5512441 A	30-04-1996
			AU	4157696 A	06-06-1996
			CA	2205392 A	23-05-1996
			WO	9615139 A	23-05-1996